

**BIOCHEMICAL AND PHARMACOLOGICAL
CHARACTERISATION OF THE INTERACTION
BETWEEN NMDA RECEPTORS AND THE
SCAFFOLDING PROTEIN, PSD-95**

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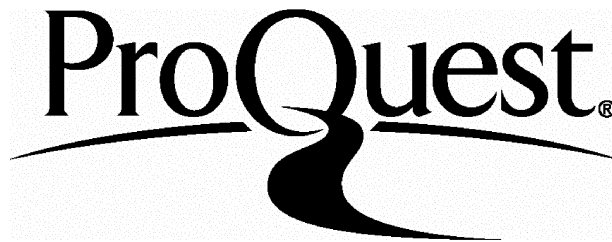
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Abstract

The N-methyl-D-aspartate (NMDA) subtype of excitatory, ionotropic L-glutamate neurotransmitter receptor is a cation-selective, ligand-gated ion channel composed of NR1 and NR2 subunits. NMDA receptors are localised on the dendritic spines of neurons where they are tightly associated with proteins of the post-synaptic density such as the scaffolding protein, post-synaptic density 95 (PSD-95). Many studies have characterised the pharmacological properties of NMDA receptors expressed in non-neuronal cell lines which lack the proteins of the post-synaptic density. The aim of this thesis was to investigate the effect of PSD-95 on the biochemical and pharmacological properties of NMDA receptors following their co-expression in mammalian cells. Various homomeric and heteromeric combinations of NR1-1a, NR1-2a, NR1-4b, NR2A and NR2B subunits were expressed $\pm$ PSD-95 in human embryonic kidney 293 cells and analysed by quantitative immunoblotting, radioligand binding and immunoprecipitation. It was found that PSD-95 both enhanced the level of NR2A and NR2B subunit expression by approximately three-fold and induced a three-fold increase in the [^{3} H]MK801 radioligand binding sites for NR1-1a/NR2A receptors. PSD-95 also increased the EC₅₀ values for the L-glutamate and glycine enhancement of [^{3} H]MK801 binding to NR1-1a/NR2A receptors by four-fold and six-fold respectively. PSD-95 however, had no effect on the affinity of NR1-1a/NR2A receptors for L-glutamate and glycine as determined by [^{3} H]CGP39653 and [^{3} H]MDL105-519 displacement assays respectively suggesting that the reduced EC₅₀ may be due to decreased channel gating. Deletion studies showed that both the biochemical and pharmacological effects of PSD-95 on expressed NMDA receptors were dependent upon the NR2 C-terminal ESDV motif, the PSD-95-binding domain. Further, mutagenesis of the two N-terminal cysteine residues of PSD-95 demonstrated that PSD-95/NMDA receptor clustering is necessary for the changes in NMDA receptor properties induced by PSD-95. These results suggest that the interaction between PSD-95 and native NMDA receptors may be important for the regulation of both receptor function and receptor number at post-synaptic sites.

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List of Abbreviations

ACBC	1-aminocyclobutanecarboxylic acid
AKAP	A kinase anchoring protein
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BEGAIN	Brain-enriched guanylate kinase-associated protein
BPPD	4-(<i>P</i> -phenyl)benzoyl piperazine dicarboxylic acid
CaMK-II	Ca ²⁺ /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CAPON	Carboxy-terminal PDZ ligand of nNOS
CGP 61594	($\pm$)- <i>Trans</i> -4-[2-(4-azidophenyl)acetylamino]-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-carboxylic acid
CGP 39653	($\pm$)-(E)-2-amino-4-propyl-5-phosphonopentanoic acid
CGS 19755	<i>Cis</i> -4-phosphonomethyl-2-piperidine carboxylic acid
Chapsyn-110	Channel-associated protein of synapses-110
7-CK	7-chlorokynurenate, <i>i.e.</i> 7-chloro-4-hydroxyquinoline-2-carboxylic acid
CNS	Central nervous system
CP-101,606	(1 <i>S</i> ,2 <i>S</i>)-1-(4-Hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol
CPP	3-(($\pm$)-2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
CREB	cAMP response element binding protein
CRIP1	Cysteine-rich interactor of PDZ three
BSA	Bovine serum albumin
DA10	1,10-Decanediamine
DAA	D- α -amino-adipate
DAP5	D(-)-2-amino-5-phosphonopentanoic acid
5, 7-DCKA	5,7-Dichlorokynurenine acid, <i>i.e.</i> 5,7-dichloro-4-hydroxyquinoline-2-carboxylic acid
dlg	Discs large
DMEM/F-12	Dulbeccos Modified Eagle Medium/Nutrient F-12 Ham
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ssDNA	Single stranded DNA
DNQX	6,7-Dinitroquinoxaline-2,3-dione
dNTP	Deoxynucleoside-5'-triphosphates

dATP	Deoxy-adenosine-5'-triphosphate
dTTP	Deoxy-thymosine-5'-triphosphate
dGTP	Deoxy-guanosine-5'-triphosphate
dCTP α S	Deoxy-cytidine-5'-[α S]thiotriphosphate
DTT	Dithiothreitol
EAA	Excitatory amino acid
EDTA	Ethylene diamine tetraacetate
EGTA	Ethylene glycol bis (β -aminoethyl ether)- <i>N, N, N', N'</i> -tetraacetate
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
EPSC	Excitatory post-synaptic current
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GABA	γ -Amino-butyric acid
GAP	GTPase-activating protein
GEP	GDP-GTP exchange protein
GFP	Green fluorescent protein
GKAP	Guanylate kinase-associated protein
GK	Guanylate kinase
GMP	Guanosine monophosphate
HA-966	R(+)-3-amino-1-hydroxypyrrolidin-2-one
HEK	Human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IPTG	Isopropylthio- β -D-galactosidase
K _D	Dissociation constant
K _I	Inhibition constant
L-687,414	(3R, 4R)-3-amino-1-hydroxy-4-methylpyrrolidin-2-one
L-689,560	<i>trans</i> -2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline
L-701,324	7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(H)quinolinone
LY-293558	(3S,4aR,6R,8aR)-6-[2-(1(2)0H-tetrazole-6-yl)ethyl]decahydroisoquinoline-3-carboxylic acid
L3	Lobe 3
LAOBP	lysine/arginine/ornithine binding protein
LB	Luria-Bertani

LDH	Lactate dehydrogenase
LGIC	Ligand-gated ion channel
LTD	Long-term depression
LTP	Long-term potentiation
nAChR	Nicotinic acetylcholine receptor
MAGUIN	Membrane-associated guanylate kinase-interacting protein
MAGUK	Membrane-associated guanylate kinase
MAP1a	Microtubule-associated protein 1a
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
MK801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
MDL 105,519	(E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid
nAChR	Nicotinic acetylcholine receptor
Narp	Neuronal activity-regulated pentraxin
NF-L	Light neurofilament
NMDA	N-methyl-D-aspartate
MOPS	3-N-[Morpholino]propane-sulphonic acid
NO	Nitric oxide
nNOS	Nitric oxide synthase
NS-102	6,7,8,9-tetrahydro-5-nitro-1H-benzo[G]indole-2,3-dione-3-oxime
NSF	N-ethylmaleimide-sensitive fusion protein
OD	Optical density
P4S	Piperidine-4-sulphonic acid
PBS	Phosphate-buffered saline
PCP	Phencyclidine, <i>i.e.</i> 1-(1-phenylcyclohexyl)piperidine
PCR	Polymerase chain reaction
PDZ	PSD-95/Dlg/ZO-1
PICK1	Protein interacting with C kinase
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PMSF	Phenylmethyl sulphonyl fluoride
PP	Protein phosphatase
PSD	Post-synaptic density
PSD-95	Post-synaptic density-95
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
QBP	Glutamine binding protein

RF	Replicative form
Ro-25-6981	[R-(R*S*)]- α -(4-hydroxyphenyl)- β -methyl-4-(phenylmethyl)-1-piperidinepropanol
RT	Room temperature
SAP	Synapse-associated protein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH3	Src homology 3
S-SCAM	Synaptic scaffolding molecule
SynGAP	Synaptic GTPase-activating protein
TBE	Tris-buffered EDTA
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TCP	N-[1-(2-thienyl)cyclohexyl]-piperidine
TEMED	<i>N, N, N', N'</i> -Tetramethylethylenediamine
THIP	4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol
TMD	Transmembrane domain
TPA	Phorbol 12-myristate 13-acetate
X-Gal	5-Bromo-4-chloro-3-indoyl- β -D-galactosidase
ZO-1	Zonula occludens

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CHAPTER 1

INTRODUCTION

1.1 L-GLUTAMATE AS A NEUROTRANSMITTER

The excitatory amino acid (EAA), L-glutamate, is now widely accepted as the mediator of fast excitatory neurotransmission in the mammalian central nervous system (CNS). The elucidation of its role in the CNS began when L-glutamate and L-aspartate were found to be potent convulsants upon direct application to the cerebral cortex of monkeys (Hayashi, 1952). The possibility of L-glutamate as a neurotransmitter was raised again when Curtis *et al.* (1959) demonstrated direct excitatory effects of L-glutamate on single neurons using the technique of microelectrophoresis. However, this proposed function of L-glutamate in synaptic transmission was met with much scepticism at this time due to the ubiquitous nature of its excitatory action on neuronal membranes. The role of L-glutamate in cell metabolism had already been established and the suggestion that it mediated excitatory effects in the brain and spinal cord may have been somewhat surprising. Nevertheless, the foundations for research in the field of excitatory amino acids had been laid. During the following 20 years, several lines of convincing evidence led to the general acceptance of L-glutamate as the major neurotransmitter in mammalian brains. Firstly, neurons in different parts of the CNS exhibited varying sensitivities to L-glutamate and L-aspartate (McLennan *et al.*, 1968), an observation inconsistent with a role of L-glutamate purely in cell metabolism. In 1974, Curtis and Johnson demonstrated that concentrations of L-glutamate and L-aspartate were relatively high in dorsal root afferents and spinal interneurons again suggesting an uneven distribution of both amino acids. The acceptance of L-glutamate as a neurotransmitter at this time required evidence of an appropriate transport pathway for the removal of L-glutamate from the extracellular space. This was provided by the identification of high affinity uptake systems for both L-glutamate and L-aspartate (Logan and Snyder, 1971; Balcar and Johnston, 1972). Further progress was made when it was shown that stimulation of afferent pathways *in vivo* resulted in the release of L-glutamate from brain tissue and that this release was partially dependent upon calcium (Jasper and Koyama, 1969; Bradford *et al.*, 1973). Finally, high affinity binding sites for [¹⁴C]L-glutamate were identified on neuronal membranes in the CNS (Michaelis *et al.*, 1974).

These initial developments sparked the growth of what is now one of the fastest moving areas of neuroscience. The synthesis of glutamate receptor antagonists and agonists fuelled the identification of pharmacologically distinct receptor subtypes that mediated glutamate-induced synaptic currents and allowed excitatory amino acid transmission to be

dissected in greater detail. The 1990s saw an explosion of interest in L-glutamate neurotransmitter receptors following the technological advances in molecular cloning. The molecular basis of receptor diversity has now been extensively characterised and has contributed further to the understanding of L-glutamate as a neurotransmitter. L-glutamate is now known to mediate the majority of fast excitatory synaptic transmission and to play an important role in the processes responsible for synaptic plasticity, memory formation and learning, and brain development. The release of excess L-glutamate in pathological conditions such as stroke and epilepsy is known to contribute to the neurodegeneration and cell death that accompanies these disease states. Consequently, glutamate receptors have been the targets for a number of therapeutic compounds designed to prevent L-glutamate-mediated excitotoxicity.

1.2 IONOTROPIC L-GLUTAMATE RECEPTORS

1.2.1 Identification of L-glutamate receptor subtypes

The first agonists identified that mimicked the excitatory actions of L-glutamate were *N*-methyl-D-aspartate (NMDA), quisqualate and kainic acid. The substitution of a methyl on the amino group of aspartate resulted in a compound, NMDA, that was more potent than its parent amino acid in eliciting post-synaptic excitation (Curtis and Johnston, 1974). Quisqualate and kainic acid were natural compounds purified from plants and seaweed respectively that exhibited excitatory effects similar to L-glutamate (Shinozaki and Shibuya, 1974, Johnston *et al.*, 1974). These compounds together with a variety of amino acids showed divergent potencies on subsets of neurons raising the hypothesis of the existence of multiple excitatory amino acid receptor classes (McLennan *et al.*, 1968; Duggan, 1974). The development of subtype-selective antagonists aided the pharmacological separation of these distinct receptor subtypes. The antagonists, D- α -amino-adipate (DAA), R(+)-3-amino-1-hydroxypyrrolidin-2-one (HA-966) and Mg^{2+} inhibited NMDA-evoked excitation with varying degrees of potency and selectivity but supported the existence of a separate class of L-glutamate receptor, *i.e.* the NMDA receptor (McLennan and Lodge, 1979; Davies and Watkins, 1979). The hypothesis that non-NMDA receptors were further subdivided into kainate and quisqualate preferring receptors was consolidated using the agonist α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) that had a distinct pharmacological profile to kainate (Krogsgaard-Larsen *et al.*,

1980). The three proposed L-glutamate receptor subtypes were therefore named after the prototypical agonists NMDA, quisqualate and kainate (Watkins and Evans, 1981; Foster and Fagg, 1984). A further alteration to the pharmacological classification of L-glutamate receptors was made following the discovery that quisqualate was a non-selective agonist that activated both the G-protein-linked metabotropic L-glutamate receptors and the AMPA-sensitive, ionotropic receptors (Sladeczek *et al.*, 1985). The non-NMDA receptors were therefore renamed AMPA and kainate receptors (Collingridge and Lester, 1989; Monaghan *et al.*, 1989). However, the distinction between AMPA and kainate receptors proved to be more complex than that of two populations of pharmacologically separable receptors. The displacement of [³H]AMPA at the high affinity site by kainate suggested some overlap between the actions of these two agonists (reviewed in Lodge, 1997). The development of more selective AMPA antagonists such as 6,7-dinitroquinoxaline-2,3-dione (DNQX) and (3S,4aR,6R,8aR)-6-[2-(1(2)0H-tetrazole-6-yl)ethyl]decahydroisoquinoline-3-carboxylic acid (LY-293558), and potential kainate receptor antagonists, such as 6,7,8,9-tetrahydro-5-nitro-1H-benzo[G]indole-2,3-dione-3-oxime (NS-102), aided the pharmacological characterisation of the AMPA- and kainate-mediated responses in rat brains (Honore *et al.*, 1988, Ornstein *et al.*, 1993, Johansen *et al.*, 1993).

1.2.2 The NMDA receptor

The NMDA receptor subtype of EAA receptor is a ligand-gated, cation-selective ion channel. Its function is typified by the action of the selective agonist, NMDA. The electrophysiological response mediated by NMDA receptors shows characteristic slow kinetics of channel activity compared to the fast kinetics of the non-NMDA L-glutamate receptors. As a result, NMDA receptor antagonists reduce the later component of synaptic events following electrical stimulation of input pathways.

The NMDA receptor possesses a number of unique properties that are fundamental to its role in neurophysiological systems. Activation of the receptor requires the simultaneous binding of L-glutamate and the obligatory co-agonist, glycine (Kleckner and Dingledine, 1988). However, ligand binding must occur in conjunction with depolarisation of the post-synaptic membrane. The patch-clamp studies of Nowak *et al.* (1984) and the voltage-clamp studies of Mayer *et al.* (1984) demonstrated that Mg²⁺ blocked the ion channel voltage-dependently giving rise to the characteristic non-linear current/voltage relationship of the NMDA receptor. This is in direct contrast to the linear

I/V relationship displayed by AMPA receptors. Thus, at hyperpolarized potentials (-30 mV to -80 mV), very little current flows through the NMDA receptor ion channel but as the post-synaptic membrane becomes depolarized, the Mg^{2+} block is removed and the current increases. This voltage-dependent block by Mg^{2+} gives the NMDA receptor its most important physiological feature and has implicated the NMDA receptor as a coincidence detector in the cellular processes of learning and memory.

Once activated, the NMDA receptor is permeable not only to Na^+ and K^+ , but also to Ca^{2+} . Mayer and Westbrook (1987) calculated that the channel was up to 10 times more permeable to Ca^{2+} than Na^+ . This is in stark contrast to the non-NMDA receptor ion channels which are generally impermeable to Ca^{2+} . The flow of Ca^{2+} ions through the activated NMDA receptor channel has important functional consequences due to the ubiquitous cell signalling properties of Ca^{2+} .

The NMDA receptor contains multiple sites of regulation which will be discussed in greater detail in section 1.2.3. In addition to the L-glutamate, glycine and Mg^{2+} recognition sites, the dissociative anesthetics, ketamine and phencyclidine (PCP), are non-competitive NMDA antagonists and define a regulatory site within the ion channel (Lodge and Anis, 1982; Anis *et al.*, 1983). Other modulatory sites exist for polyamines, Zn^{2+} , protons, ethanol and cannabinoids. The NMDA receptor is also subject to redox modulation and is regulated by phosphorylation of specific intracellular amino acid residues.

1.2.3 NMDA receptor pharmacology

The glutamate recognition site

The site of recognition for the endogenous agonist, L-glutamate, has been a successful target for the synthesis of selective agonists and antagonists of the NMDA receptor. In addition to the L-glutamate analogues, NMDA and DL-homocysteate, other synthetic compounds including tetrazol-5-ylglycine (Schoepp *et al.*, 1991) and some α -carboxycyclopropylglycines (Shinozaki *et al.*, 1989) bind at the L-glutamate site with higher affinity.

The development of the highly potent and selective NMDA antagonist D(-)-2-amino-phosphonopentanoic acid (DAP5), a derivative of DAA, aided the classification of L-glutamate receptors into distinct pharmacological entities (Davies *et al.*, 1981; Watkins *et al.*, 1990). Other compounds displaying higher affinities have been

developed including 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; Olverman *et al.*, 1989), *cis*-4-phosphonomethyl-2-piperidine-2-carboxylic acid (CGS 19755; Lehmann *et al.*, 1988) and (±)-(E)-2-amino-4-propyl-5-phosphonopentanoic acid (CGP 39653; Sills *et al.*, 1991), and are invaluable tools for the pharmacological characterisation of NMDA receptors by radioligand binding and autoradiographic studies.

The binding of both L-glutamate site agonists and antagonists is subject to allosteric modulation by the binding of various ligands at other recognition sites, in particular the glycine site. The affinity of L-[³H]glutamate for its binding site is generally increased by glycine site agonists and decreased by glycine site antagonists, for example, (HA-966) and 1-aminocyclobutanecarboxylic acid (ACBC) (Fadda *et al.*, 1988; Monaghan *et al.*, 1988; Compton *et al.*, 1990; Kemp and Priestley, 1991). This effect of glycine is consistent with its role as a co-agonist and demonstrates allosteric coupling between the L-glutamate and glycine binding sites. The binding of the L-glutamate site antagonists, [³H]CGS 19755 and [³H]CGP 39653, both analogues of AP5, is negatively regulated by glycine (Kaplita and Ferkany, 1990; Mugnaini *et al.* 1993; Monaghan *et al.*, 1988). In the case of [³H]CGP 39653, this has been shown to be due to a glycine-induced increase in the dissociation kinetics of [³H]CGP 39653 binding. However, in contrast to these L-glutamate antagonists, the binding of the antagonist, CPP, is positively modulated by glycine (Porter *et al.*, 1992) suggesting that the allosteric interactions between the L-glutamate and glycine sites is highly complex.

The glycine recognition site

In 1987, Johnson and Ascher showed that glycine facilitated the action of L-glutamate on the NMDA receptor channel. Subsequently, Kleckner and Dingledine (1988) showed that glycine was absolutely necessary for the activation of the NMDA receptor channel by NMDA or glutamate. However, the concentration of glycine in the extracellular space of the CNS has been reported to be in the micromolar range, well above the dissociation constant for the binding of glycine to the NMDA receptor (100 - 200 nM; Ascher and Johnson, 1994). In a study by Fletcher *et al.* (1989), glycine did not potentiate the actions of NMDA in intact tissues suggesting that the extracellular levels of glycine were close to saturating. However, in a more recent molecular study, by co-expressing the glycine transporter (GLYT) 1 with NMDA receptors in *Xenopus* oocytes it was shown that very efficient buffering of local glycine concentration exists, leading to a >100-fold concentration gradient (Supplisson and Bergman, 1997). Thus, the concentration of glycine

at the synapse may well be below its K_D if the glycine transporter is expressed sufficiently at the right location *in vivo*.

The early studies characterising NMDA receptor pharmacology revealed that the actions of glycine on the NMDA receptor were mimicked by D-serine, but were not blocked by strychnine which is an antagonist of inhibitory glycinergic neurotransmission. The glycine site of the NMDA receptor was therefore termed the strychnine-insensitive glycine site. The identification that glycine was a co-agonist for NMDA-mediated excitatory actions explained the characteristics of a number of NMDA receptor antagonists which were known to display non-competitive kinetics. It was subsequently found that HA-966, kynurenate and 7-chlorokynurenate (7-CK) all compete for the glycine site rather than the L-glutamate site (Kessler *et al.*, 1989; Fletcher and Lodge, 1988; Kemp *et al.*, 1988). Allosteric coupling between the L-glutamate and glycine binding sites has also been demonstrated at the glycine site (reviewed in Danysz and Parsons, 1998). For example, glutamate increases the affinity of cortical NMDA receptors for glycine and D-serine (Nguyen *et al.*, 1987; Ransom and Deschenes, 1990).

The 1990s saw the generation of a number high affinity selective antagonists at the glycine site. These include 5, 7-dichloro-kynurenate (5,7-DCKA), *trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline (L-689,560; Foster *et al.*, 1992) and (E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid (MDL 105,519; Baron *et al.*, 1996).

Ion channel blockers

Another class of NMDA receptor antagonist identified in the 1980s was the defined by the action of the dissociative anaesthetics, PCP and ketamine (Anis *et al.*, 1983). These compounds antagonised NMDA responses of rat spinal neurons but their mode of action was not consistent with activity at either the L-glutamate or glycine recognition sites. Instead, ketamine and PCP induced a non-parallel rightward shift in the dose-response curve for NMDA-induced currents suggesting a non-competitive mechanism of action (Snell and Johnson, 1985; Lodge and Johnson, 1985). NMDA receptor agonists and PCP-like compounds did not displace each others binding discounting the L-glutamate recognition site as the target for these compounds. Furthermore, the action of the ketamine (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK801) was shown by electrophysiological and radioligand binding studies to be use-dependent and voltage-dependent (Foster and Wong, 1987; Davies *et al.*, 1988). It was

therefore proposed that this class of NMDA antagonist bound to the open channel of the NMDA receptor.

MK801 is an anti-convulsant with high potency and selectivity for the NMDA receptor (Wong *et al.*, 1986; Wong *et al.*, 1988). The high affinity of MK801 for its site has permitted the study, by radioligand binding, of the allosteric effects of other regulatory molecules on channel activation. Ransom and Stec (1988) elegantly demonstrated the use-dependency of MK801 binding by measuring the allosteric enhancement of [^3H]MK801 binding by L-glutamate and glycine. Glutamate and glycine were shown to mediate their effects concentration dependently by increasing the affinity of the receptor for [^3H]MK801. Indeed, the modulation by both these agonists was cooperative, demonstrating allosteric coupling between the L-glutamate and glycine sites. The modulation of [^3H]MK801 binding by other regulatory molecules is consistent with their observed effects on NMDA responses *in vivo*. Thus, glycine site antagonists, glutamate site antagonists, and Mg^{2+} all inhibit the binding of MK801 due to their effects on channel opening. In addition, the polyamines spermine and spermidine enhance MK801 binding (discussed below).

Modulation by divalent cations

The non-competitive block of NMDA responses by Mg^{2+} was initially documented by Evans *et al.* (1977). Subsequently, the voltage dependence of the Mg^{2+} block was shown to be responsible for the decreased NMDA-mediated current responses at hyperpolarized neuronal membrane potentials (Nowak *et al.*, 1984). NMDA receptor channels are also blocked by physiological concentrations of intracellular Mg^{2+} in a voltage-dependent manner (Johnson and Ascher, 1990). In addition to Mg^{2+} , the divalent cations Ni^{2+} , Mn^{2+} , Co^{2+} , Zn^{2+} , and to a lesser extent Ca^{2+} , all antagonise NMDA responses (Evans *et al.*, 1977; Ault *et al.*, 1980).

Zn^{2+} accumulates at some nerve terminals in specific brain regions and it is released into synaptic clefts in a Ca^{2+} -dependent manner during neuronal activity (Smart *et al.*, 1994). At low concentrations (1-10 μM), Zn^{2+} has been shown to antagonise NMDA responses in cultured neurons although its blocking action is not voltage-dependent and is thought to be a result of a reduced channel open time (Peters *et al.*, 1987; Westbrook and Mayer, 1987; Mayer *et al.*, 1989). At higher concentrations, Zn^{2+} additionally produces voltage-dependent inhibition of NMDA responses (Christine and Choi, 1990). Zn^{2+} also inhibits glutamate uptake and potentiates AMPA receptors suggesting that it may be an important neuromodulator of ion channels *in vivo* (Spiridon *et al.*, 1998).

Modulation by protons

NMDA receptor channels are inhibited by protons with an IC_{50} value close to physiological pH, implying that NMDA receptor channels are tonically inhibited under normal conditions (Tang *et al.*, 1990; Traynelis and Cull-Candy, 1990).

Modulation by polyamines

The endogenous polyamines spermine and spermidine are present in high concentrations in the CNS and depolarization-induced release of polyamines from brain slices and their subsequent uptake has been reported (Harman and Shaw, 1981). The first demonstration that NMDA receptors were modulated by polyamines was by Ransom and Stec (1988). They discovered that the polyamines, spermine and spermidine, enhanced the glycine-induced binding of [^{3} H]MK801 to rat cerebral cortex membranes. Since then, it has been shown that polyamines modulate the NMDA receptor by four distinct mechanisms. At sub-saturating concentrations of glycine, polyamines potentiate NMDA receptors by increasing the apparent affinity of the receptor for glycine (Sacaan and Johnson, 1989; Benveniste and Mayer, 1993). At saturating concentrations, polyamines stimulate receptor opening at concentrations below 200 mM by increasing open channel frequency (Williams *et al.*, 1990; Rock and MacDonald, 1992a; Lynch *et al.*, 1995), whereas at even higher concentrations, polyamines decrease the affinity of the receptor for glutamate (Williams *et al.*, 1995) and block NMDA receptors in a voltage-dependent manner by decreasing channel amplitude and average open time (Rock and MacDonald, 1992a; Rock and MacDonald, 1992b; Benveniste and Mayer, 1993). Polyamines have therefore been classified as agonists, partial agonists, antagonists and inverse agonists (Williams *et al.*, 1990), and their actions subdivided into glycine-dependent and glycine-independent mechanisms. An interpretation of these diverse and apparently opposing modulatory effects was that two distinct polyamine recognition sites exist within the NMDA receptor complex (Rock and MacDonald, 1992a; Benveniste and Mayer, 1993), one site allosterically coupled to the glycine recognition site and another within the ion channel controlling the voltage-dependent inhibition of NMDA responses. Polyamines did not displace [^{3} H]CCP, [^{3} H]glycine or [^{3} H]MK801 binding from NMDA receptors (Ransom and Stec, 1988). Thus, the site of action of polyamines is distinct from the L-glutamate, glycine and channel blocking sites.

The pharmacology of polyamine antagonists is similarly complex. The high affinity antagonists, arcaine and 1,10-Decanediamine (DA10), inhibited some but not all

effects of polyamines, again suggesting multiple polyamine sites (arcaine, Reynolds, 1990a; Reynolds, 1990b; DA10, Williams *et al.*, 1990). The NMDA receptor antagonist, ifenprodil, was initially thought to act at the polyamine site (Carter *et al.*, 1990). However, other groups found that ifenprodil acts at a distinct site, although the polyamine site and the ifenprodil binding sites may be allosterically coupled (Reynolds and Miller, 1989; Williams *et al.*, 1995).

Modulation of NMDA receptor function by protein phosphorylation

Protein kinases and protein phosphatases have long been known to be crucial regulators of protein function, and are key components in the biochemical events controlling many cellular processes including cell proliferation, growth and differentiation. These enzymes regulate their substrates by stimulating the phosphorylation or dephosphorylation respectively of either serine, threonine or tyrosine amino acid residues located in the target protein. This post-translational modification is a dynamically regulated process allowing the rapid activation or inhibition of cell signalling pathways. The protein kinases can be broadly categorised into serine/threonine protein kinases (which includes cAMP-dependent protein kinase [PKA], protein kinase C [PKC] and Ca^{2+} /calmodulin-dependent protein kinase II [CaMK-II]) and tyrosine kinases.

Protein kinases and phosphatases are expressed at high levels in the developing and mature central nervous system. Most L-glutamate receptors are regulated by protein phosphorylation, in particular NMDA receptors. For example, bath application of phorbol ester, a known protein kinase activator, to spinal dorsal horn slices increased both the amplitude and duration of NMDA-induced depolarizations (Gerber *et al.*, 1989). Similarly in 1992, Aniksztejn *et al.* showed that the activation of metabotropic receptors up-regulated NMDA receptor currents and that this effect was dependent on PKC activity. It has been proposed that PKC activity modulates NMDA receptors by both increasing the probability of channel opening and relieving the voltage-dependent Mg^{2+} block of these channels (Chen and Huang, 1992). Intriguingly, the treatment of neurons with phorbol esters sometimes induced a depression in NMDA receptor currents (Markram and Segal, 1992; Courtney and Nicholls, 1992). NMDA receptors are also subject to regulation by PKA which in contrast to PKC is activated by cyclic-AMP (Raman *et al.*, 1996). PKC enzyme isoforms are generally activated by both diacylglycerol, generated by the hydrolysis of phospholipids, and Ca^{2+} .

The NMDA receptor is also regulated by protein phosphatases. This is not

surprising since the steady state level of phosphorylation is likely to be controlled by the relative activities of kinases and phosphatases. Wang *et al.* (1994) noted an enhancement of NMDA currents in cultured hippocampal neurons following application of calyculin A, an inhibitor of serine/threonine protein phosphatase 1 (PP1) and PP2A, consistent with the potentiation of current responses due to enhanced phosphorylation of NMDA receptors. Similarly, direct application of PP1 or PP2A to inside-out patches reduced the probability of NMDA channels opening.

The regulation of NMDA receptor function by protein tyrosine kinases (PTKs) and phosphatases (PTPs) has also been characterised. A functional role for the tyrosine phosphorylation of NMDA receptors was demonstrated by the perfusion of spinal neurons with the PTK inhibitors, genistein and lavendustin. Both of these inhibitors suppressed NMDA receptor currents, while perfusion with an activated form of the tyrosine kinase, src, potentiated currents (Wang and Salter, 1994). Similarly, Yu *et al.* (1997) measured a src-mediated increase in NMDA channel open probability measured in patches excised from mammalian central neurons. Conversely PTP activity in neuronal cells was shown to decrease the channel open probability (Wang *et al.*, 1996).

1.2.4 Heterogeneity of native NMDA receptors

The early pharmacological and electrophysiological studies suggested NMDA receptors were functionally heterogeneous. In 1983, Perkins and Stone noted that the NMDA receptor agonist, quinolinic acid, did not elicit excitatory responses in the cerebellum and spinal chord despite observing L-glutamate-induced excitatory responses in these areas. Further results from both radioligand and electrophysiological studies have identified four pharmacologically distinct populations of NMDA receptor. A broad division in NMDA receptor properties can be made between forebrain and cerebellar receptors based on their distinct pharmacological profiles at both the L-glutamate and channel blocker sites. Forebrain receptors can be further subdivided on the basis of agonist and antagonist selectivity. The fourth population of NMDA receptor found in the medial thalamic nuclei displays a pharmacological profile very similar to cerebellar receptors but demonstrates some additional antagonist selectivity.

Cerebellar NMDA receptors

In general, both L-glutamate site antagonists and open channel site antagonists are significantly less potent to cerebellar membranes compared to forebrain with the exception of remacemide which displays a higher affinity for cerebellar receptors (Vignon *et al.*, 1986; Quaram *et al.*, 1990; Ebert *et al.*, 1991; Yoneda and Ogita, 1991; Beaton *et al.*, 1992; Porter and Greenamyre, 1995). The affinity of cerebellar membranes for [³H]MK801 in particular is significantly reduced. In contrast to the decreased affinity of cerebellar receptors for L-glutamate site antagonists, L-glutamate site agonists display similar affinities for both receptor populations. There is evidence to suggest however, that cerebellar receptors have a decreased sensitivity to glycine compared to forebrain NMDA receptors (O'Shea *et al.*, 1991; Monaghan *et al.*, 1988; Sekiguchi *et al.*, 1990). In addition, the polyamine, spermine, potentiates [³H]MK801 binding to forebrain membranes but has little effect on [³H]MK801 binding to cerebellar NMDA receptors (Yoneda and Ogita, 1991).

NMDA receptors in the medial thalamic nuclei

NMDA receptors located in the medial thalamic nuclei display similar reduced affinities for glutamate site antagonists and channel blockers compared to forebrain receptors. However, a subpopulation of NMDA receptors pharmacologically distinct from cerebellar NMDA receptors was identified in the medial thalamic nuclei using the glutamate site antagonist, LY-23536, which was a relatively weak displacer of [³H]glutamate from cerebellar membranes but relatively potent in the medial thalamic nuclei (Beaton *et al.*, 1992). The non-competitive channel blocking antagonists, TCP and SKF-10047, also display a distinct pharmacological profile in the medial thalamic nuclei compared to both forebrain and cerebellar NMDA receptors (Beaton *et al.*, 1992).

Forebrain NMDA receptors

The two remaining distinct subpopulations of NMDA receptor located in mammalian forebrains are defined by their relative affinities for agonists and antagonists and by their sensitivity to polyamines. Most NMDA receptors in the forebrain display similar pharmacological profiles for agonists and antagonists such that forebrain NMDA receptors are clearly distinct from both cerebellar and medial thalamic nuclei NMDA receptors. However, heterogeneity in receptor function has been identified in certain regions of the forebrain. For example, receptors located in the striatum are defined as

agonist-preferring receptors owing to their higher relative sensitivity to agonists compared to receptors located in the thalamus (Monaghan, 1991). Conversely, receptors in the thalamus display a higher relative sensitivity to antagonists compared to the striatum and are thus defined as antagonist-preferring receptors (Monaghan *et al.*, 1988; van Amsterdam, 1992; Sakurai *et al.*, 1993). Additional regional differences in the sensitivity of [³H]MK801 binding to polyamines have been identified. Thus, agonist-preferring receptors are also more sensitive to potentiation by spermine compared to antagonist preferring receptors (Subramaniam and McGonigle, 1991).

1.3 CLONING OF THE IONOTROPIC L-GLUTAMATE RECEPTOR GENES

The cloning of NMDA receptor subunit genes was a long awaited event and dramatically accelerated the understanding of receptor function providing new insights into the pharmacological and electrophysiological characteristics of the NMDA receptor. However, the first glutamate receptor subunit cloned by expression cloning in *Xenopus* oocytes was in fact the GluR1 subunit of the AMPA receptor (Hollmann *et al.*, 1989). This was followed rapidly by the cloning of a further nine AMPA/kainate receptors by standard homology screening and polymerase chain reaction (PCR)-mediated screening (reviewed in Hollmann and Heinemann, 1994). The genes GluR1-4 comprise the AMPA receptor subunit family whereas the gene, GluR5-7 and KA 1-2, comprise the kainate receptor subunit family. The polypeptides within each family share approximately 70 % amino acid sequence homology, whereas between each family they share approximately 40 % homology. The cloning of a further 8 glutamate receptor genes, including the NMDA receptor genes, extended the ionotropic glutamate receptor family to 17 genes (summarised in Figure 1.1).

The first cDNA sequence encoding an NMDA receptor subunit was isolated from rat brain using expression cloning and was named NMDAR1 (Moriyoshi *et al.*, 1991). The nucleotide sequence shows an open-reading frame of 2,814 nucleotides encoding a protein of 938 amino acids with predicted $M_r = 105,500$ Da. The sequence displays 22-26 % identity to the GluR1 subunit. Further cloning by other laboratories identified 7 alternatively spliced isoforms of the NR1 subunit encoding polypeptides of 883-941 amino acids with predicted $M_r = 97,300$ -105,000 Da (Durand *et al.*, 1992; Nakanishi *et al.*, 1992; Sugihara *et al.*, 1992; Yamazaki *et al.*, 1992a, Hollmann *et al.*, 1993). The subsequent

determination of the chromosomal localization of NR1 revealed that the NR1 gene contains 22 exons, three of which undergo alternative splicing to generate theoretically eight splice variants. Exon 5 encodes a splice cassette of 21 amino acids, termed N1, that is inserted in the amino-terminus domain at residue 190. An unusual feature of the N1 cassette is that 9 of the 21 residues are charged amino acids. Exons 21 and 22 encode two independent consecutive cassettes of 37 (C1 cassette) and 38 (C2 cassette) amino acids, respectively, that constitute the last stretch of the C-terminus. Splicing out of exon 22 removes the first stop codon resulting in a new open-reading that encodes an unrelated sequence of 22 amino acids termed C2'. The splice variants are represented schematically in Figure 1.2 and are labelled according to the nomenclature derived by Nakanishi *et al.* (1992). The equivalent NR1 gene was also isolated from both mouse (NR ζ 1) and human (hNR1) showing 99 % amino acid sequence identity to the rat gene with amino acid variations at only two residue positions in the mouse (Yamazaki *et al.*, 1992a) and four in the human (Karp *et al.*, 1993). Initial analyses of the NR1 amino acid sequence by hydrophobicity plot identified four possible transmembrane domains (TMDs). This was analogous to the four domains found in the non-NMDA ionotropic receptor subunits. However, further topological characterisation of the NMDA receptor subunits has led to the general acceptance of a re-defined version of the four TMD model (discussed in section 1.4.1).

The first cDNA encoding an NR2 gene was isolated by low stringency screening of a mouse cDNA library using mouse AMPA/kainate cDNA probes (Meguro *et al.*, 1992) and was termed NR ϵ 1 (or NR2A in rat). This was followed by the identification of three other NR2 genes, NR2B, NR2C and NR2D (or NR ϵ -2, NR ϵ -3 and NR ϵ -4, in mouse) by screening and polymerase chain reaction amplification (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993). The predicted mature subunits for the mouse NR2A-2D subunits are composed of 1445, 1456, 1220, 1296 amino acid residues with molecular masses of 163267, 162875, 133513 and 140656 Da respectively. The NR2 amino acid sequences share ~50 % homology with each other, but display only ~15 % identity to the NR1 gene. In addition, the lengths of the NR2 subunit C-termini differ notably from the NR1 subunit by the presence of an extra 200-400 amino acids (summarised in figure 1.13). The identification of the equivalent NR2 subunits (hNR2A-D) in the human revealed that unlike the NR1 gene, the NR2 genes display less cross-species homology (Le Bourdelles *et al.*, 1994; Hess *et al.*, 1996; Lin *et al.*, 1996). Thus, the human NR2A differs from the rat amino acid sequence at 81 amino acids, and the human and mouse NR2C subunits display 88 % and 95 % amino acid homology to the rat NR2C

sequence respectively.

Immediately following the isolation of the first receptor subunit clone, NMDA receptors were expressed in recombinant systems to investigate whether functional NMDA receptors could be reconstituted *in vitro*. The very nature of the method used to clone the first NMDA receptor implied that homomeric NMDA receptors composed of a single NR1 subunit could form functional NMDA receptors that displayed similar pharmacological characteristics to native NMDA receptors (Moriyoshi *et al.*, 1991). Homomeric NR1-1a receptors expressed in *Xenopus* oocytes by the injection of mRNA were activated by L-glutamate and the co-agonist, glycine, modulated by Zn^{2+} , blocked voltage-dependently by Mg^{2+} and were permeable to Ca^{2+} . The pharmacology of L-glutamate site antagonists, glycine site antagonists and channel blockers was consistent with their inhibitory properties on native NMDA receptors (Moriyoshi *et al.*, 1991; Yamazaki *et al.*, 1992a; Nakanishi *et al.*, 1992). However, expression of homomeric NR1 NMDA receptors in mammalian cell lines did not result in the reconstitution of functional NMDA receptor channels (Monyer *et al.*, 1992; Lynch *et al.*, 1995). Even though NR1-1a subunit immunoreactivity could be detected in transfected human embryonic kidney (HEK) 293 cells, the specific binding of [^{125}I]MK-801 and [^3H]CGP 39653 could not be detected (Lynch *et al.*, 1994; Laurie and Seeburg, 1994). Highly active recombinant NMDA receptor channels were only reconstituted in mammalian cells upon the co-expression of an NR1 subunit and one of the four NR2 subunits (Ishii *et al.*, 1993; Monyer *et al.*, 1992; Meguro *et al.*, 1992; Kutsuwada *et al.*, 1992; Ikeda *et al.*, 1992). An explanation for this difference between the function of cloned NMDA receptors in *Xenopus* oocytes and mammalian cell lines was provided by Soloviev and Barnard (1997) who showed that a glutamate receptor subunit, XenU1, was endogenously expressed in *Xenopus* oocytes but not in mammalian cell lines. This subunit was subsequently shown to co-assemble with the NR1 subunit in mammalian cell lines resulting in the expression of functional NMDA receptors and explains why expression cloning in *Xenopus* oocytes was originally possible. It is now widely accepted that the expression of functional cloned NMDA receptors requires both NR1 and NR2 subunits.

Another cDNA clone, NR3A, encoding a 1115 amino acid polypeptide, identified in 1995 was termed NMDA-L (Sucher *et al.*, 1995) or $\chi 1$ (Ciabarra *et al.*, 1995) and was described as an NMDA receptor-like subunit owing to its 27 % amino acid identity to the NR1 subunit. Although by itself the NR3A subunit does not form functional NMDA receptor channels, co-expression of NR3A with NR1/NR2 receptors in *Xenopus* oocytes yielded smaller unitary conductances compared to NR1/NR2 receptors alone (Das *et al.*,

1998). The NR3A subunit is expressed primarily during brain development when it can be co-immunoprecipitated with NR1 and NR2 subunits. Genetic knockout of NR3A in mice resulted in enhanced NMDA responses and increased dendritic spine density in early postnatal neurons suggesting that NR3A modulates the NMDA receptor activity during development (Das *et al.*, 1998).

Following the cloning and characterisation of the AMPA, kainate and NMDA receptor subunit genes, two additional homologous genes were cloned and included in the ionotropic glutamate receptor family. Screening of a mouse forebrain library by low-stringency hybridization with GluR1-GluR4 probes led to the isolation of a cDNA clone with approximately 30 % homology to other GluR family members. This gene, termed delta1, was later joined by delta2, a gene with 56 % identity to delta1 (Yamazaki *et al.*, 1992b; Lomeli *et al.*, 1993). These two proteins do not bind glutamatergic ligands and have thus been relegated to the status of orphan receptors. However, abundant expression of delta1 and delta2 in various regions of the CNS has been demonstrated, especially in the cerebellum and auditory system (Araki *et al.*, 1993; Mayat *et al.*, 1995; Safieddine and Wenthold, 1997). The transplantation of the pore regions of either delta1 or delta2 into either GluR1, GluR6, or NR1 abolished the activity of these subunits suggesting that they do not contribute to the formation of ion channels but may instead serve some regulatory function (Villmann *et al.*, 1999).

1.4 STRUCTURE AND FUNCTION ON CLONED NMDA RECEPTORS

1.4.1 Membrane topology of the NMDA receptor

Once the new genes for the NMDA receptor subunits were cloned, the sequences were submitted for hydrophobicity plot analysis to gain some insights into the possible structure of the receptor subunits. Initially, it was thought that the L-glutamate receptor genes were members of the superfamily of evolutionarily-linked receptors, the ligand-gated ion channel (LGIC) superfamily, and the patterns obtained were reminiscent of the hydrophobicity plots of the previously cloned nicotinic acetylcholine receptors (nAChR), γ -amino-butyric acid_A (GABA) receptors (GABA_AR), glycine receptors and AMPA receptors. Since no other experimental data was available, the model of the nAChR was adopted as a guide and led to the proposal of a four transmembrane domain model with both N- and C-termini located extracellularly (Keinanen *et al.*, 1990; Nakanishi *et al.*,

1990). This model represented the generally accepted consensus topology for several years. However, as summarised in the following section, the identification of residues involved in the formation of the glutamate and glycine binding sites, redox modulation sites, N-glycosylation sites, and phosphorylation sites led to the modification of this model and furthermore, suggested that in fact that the ionotropic GluRs formed their own superfamily and were not related to other neurotransmitter receptor proteins. The accepted model for the membrane topology of the NMDA receptor subunits, summarised in Figure 1.4, now places the N-terminus extracellularly, while the C-terminus resides intracellularly, and the domain previously thought to be TMD2 forms a hair-pin loop within the membrane. The nomenclature shown in Figure 1.4, therefore describes the hydrophobic regions as membrane (M) domains rather than transmembrane domains.

Phosphorylation sites

The phosphorylation of integral transmembrane proteins is thought to occur exclusively at intracellular sites due to the activity of cellular protein kinases and phosphatases. Consequently, the identification of a phosphorylation site is regarded as proof for the cytoplasmic localization of the respective site. Tingley *et al.* (1993, 1997) demonstrated that three serine residues located in exon 21 were phosphorylated, placing the C-terminus of NR1 on the cytoplasmic side of the membrane. Mori *et al.* (1993) also showed that the modulation of NMDA receptor function by phorbol 12-myristate 13-acetate (TPA), a PKC activator was mediated by the C-terminal tail of NR2B suggesting the localization of phosphorylation sites in this region and thus an intracellular location of the NR2B subunit C-terminus. This evidence was inconsistent with the proposed four-TMD model which placed the C-terminus on the extracellular side of the membrane.

N-Glycosylation

N-Glycosylation of proteins involves the attachment of a carbohydrate side chain to asparagine residues that are located in the consensus sequence, N-X-S or N-X-T, where X is not a proline (Hart *et al.*, 1978). The glycosylation process is strictly compartmentalized so that only residues in the extracellular regions of a membrane protein are glycosylated. Therefore, glycosylation also gives clues to the membrane topology of NMDA receptors. Wood *et al.* (1995) showed that an engineered glycosylation site at the beginning of lobe 3 (L3), the domain located between the putative membrane domains two and three of NR1, was glycosylated in *Xenopus* oocytes. Furthermore, a glycosylation site

located at the end of L3 in wild-type NR1 subunit was glycosylated following expression in *Xenopus* oocytes (Wood *et al.*, 1995). This placed the entire L3 domain extracellularly. If L3 was indeed extracellular then M2 could not cross the membrane as predicted by the TMD4 model. Wood *et al.* (1995) also pointed out that there was significant sequence homology between the M2 region of NR1 and the pore region of potassium channels. The pore region of the voltage-gated K⁺ channels is a reentrant loop or hairpin structure. Wood *et al.* (1995) therefore postulated that the M2 domain of NR1 formed a hairpin loop within the membrane and returned to the cytoplasmic side of the membrane.

Ligand binding domains

As discussed in more detail in section 1.4.2, the amino acids involved in the formation of the glycine and L-glutamate binding sites were shown to reside on the N-terminal region and the domain between M3 and M4 of NR1 and NR2 subunits respectively. In addition, the two cysteine residues involved in polyamine regulation and redox modulation were localised to the domain between M3 and M4. The location of these amino acids was therefore consistent with an extracellular location of the N-terminus and L3.

Immunological localisation

The intracellular or extracellular location of NMDA receptor subunit domains was investigated with the use of either peptide specific antibodies raised against various regions of the NR1 subunit or with monoclonal antibodies raised against epitope tags positioned in the NR1 subunit polypeptide. Using antibodies raised against the NR1 C-terminus, Chazot *et al.* (1995) demonstrated an intracellular location of this region. In agreement, Hirai *et al.* (1996) showed that an epitope inserted into the C-terminus of the NR1 subunit was localised intracellularly. Furthermore, epitope tagging revealed that both the N-terminus and the L3 domain were exposed extracellularly (Hirai *et al.* 1996).

1.4.2 Protein domains involved in ligand binding and allosteric modulation

The availability of the NR1 and NR2 subunit clones has enabled the further characterisation of the protein domains responsible for the observed pharmacology of native and cloned NMDA receptors. In many cases the localisation of these recognition sites explains the observed pharmacology of the corresponding ligand or allosteric modulator,

and has aided the synthesis of NMDA receptor subtype-selective antagonists.

The glycine binding site

Molecular pharmacological and electrophysiological analyses of homomeric and heteromeric NMDA receptors have localised the glycine binding site of the NMDA receptor exclusively to the NR1 subunit. After the initial cloning of the NR1 subunit, the glycine binding site was proposed to reside on the NR1 subunit because homomeric NR1 receptors bound glycine agonists and antagonists (Moriyoshi *et al.*, 1991; Laurie and Seeburg, 1994; Grimwood *et al.*, 1995a). This proposal was substantiated by mutagenesis studies that identified residues in the NR1 subunit that reduced the efficacy of glycine but not of L-glutamate (Kuryatov *et al.*, 1994; Wafford *et al.*, 1995; Williams *et al.*, 1996; Hirai *et al.*, 1996). The glycine recognition site is now thought to be localised to two non-contiguous domains (summarised in Figure 1.5), one located in the NR1 N-terminal domain (termed S1) and one in the loop connecting M3 to M4 (S2) both of which demonstrate homology to a bacterial glutamine binding protein (QBP) and a lysine/arginine/ornithine binding protein (LAOBP; Kuryatov *et al.*, 1994). It has been proposed that a conserved amino acid binding pocket exists in all glutamate receptors and is formed from the two globular domains S1 and S2. Indeed a soluble glycine-binding site with the correct pharmacology was preserved in a fusion protein consisting of the S1 and S2 lobes of the NR1 subunit connected by a linker (Ivanovic *et al.*, 1998).

The L-glutamate binding site

Initially the L-glutamate recognition site was thought to reside on the NR1 subunit due to the greater homology of the S1 and S2 regions of NR1 to the L-glutamate binding domains of non-NMDA receptor subunits. However, high affinity binding of L-glutamate and NMDA to singly expressed NR2A subunits suggested that NR2 subunit proteins were sufficient to form proper L-glutamate binding sites (Kendrick *et al.*, 1996). Furthermore, the mutagenesis of specific amino acids residing in the region preceding M1 and the loop domain connecting M3 to M4 of NR2A and NR2B subunits significantly reduced the efficacy of L-glutamate but not glycine suggesting that the L-glutamate binding site was located on the NR2 subunit (Laube *et al.*, 1997; Anson *et al.*, 1998). These residues found on the NR2 subunit are in the homologous regions of the NR1 subunit that control glycine potency. The overall folding of the area of NR2 subunits that binds glutamate is likely therefore to be structurally similar to the area of the NR1 subunit that binds glycine.

The ion channel pore

The identification of a specific asparagine residue (termed the N-site) in the M2 domain of the NR1 subunit that controlled both the calcium permeability and Mg^{2+} voltage-dependency of the NMDA receptor, was convincing evidence that the M2 region formed the pore of the NMDA receptor ion channel (Burnashev *et al.*, 1992a; Mori *et al.*, 1992; Kawajiri and Dingledine, 1993). The equivalent site in the NR2 subunits, in conjunction with an additional asparagine residue (termed the N+1 site) adjacent to the N-site also contributed to the Mg^{2+} block site (Sakurada *et al.*, 1993). In contrast to the NR1 subunit however, mutagenesis of the N-site in NR2A had no effect on Ca^{2+} permeability. The location of this asparagine residue in the M2 domain of NR1 is analagous to the position of an arginine in the M2 domain of the AMPA receptor GluR2 subunit. While the other AMPA receptor subunits contain a glutamine at this position, the GluR2 subunit mRNA is subject to RNA editing resulting in the substitution of this glutamine for arginine (Hollmann *et al.*, 1991). This Q/R substitution was shown to be responsible for the calcium impermeability of the GluR2 subunit in contrast to the Ca^{2+} permeability of the other AMPA receptor subunits (Hume *et al.*, 1991; Burnashev *et al.*, 1992b). The contribution of the predicted M2 region of NR1 and NR2 subunits to the formation of the ion channel pore was also demonstrated by the cysteine-substitution of specific M2 amino acids that resulted in a reduced ion permeability (Kuner *et al.*, 1995). In addition, the molecular determinants that specify the block of NMDA receptors by the non-competitive antagonist, PCP, are confined to the M2 region confirming the mechanism of action of PCP as an ion channel blocker (Ferrer-Montiel *et al.*, 1995).

Regulation by protons and polyamines

Two cysteine residues in the L3 region of the NR1 subunit are involved in the proton inhibition and polyamine regulation of NMDA receptor channels (Sullivan *et al.*, 1994). Elimination of these two cysteines at amino acid positions 726 and 780 abolished potentiation by polyamines and decreased the inhibition of NMDA receptors by protons. Notably, the mutagenesis of specific residues in the N-terminal domain of both the NR1 and NR2B subunit, and in the L3 region of NR2B inhibited the effects of polyamines (reviewed in Yamakura and Shimoji, 1999). Many other residues in the M1, M2, M3 and L3 regions of NR1, and in the M2, M3 and L3 regions of NR2B were also shown to influence the modulation of NMDA receptor function by polyamines and protons (Sullivan *et al.*, 1994; Williams *et al.*, 1995; Kashiwagi *et al.*, 1996; Kashiwagi *et al.*, 1997).

1.4.3 Subunit stoichiometry of the NMDA receptor

The initial observation that the expression of fully functional cloned NMDA receptors in mammalian expression systems required an NR1 and an NR2 subunit indicated that these receptors were formed by the co-assembly of NR1 subunits with NR2 subunits. Indeed, the co-association of cloned NR1 subunits with NR2 subunits has been demonstrated by co-immunoprecipitation studies (Chazot *et al.*, 1994). However, the precise NR1:NR2 stoichiometry of both cloned and native NMDA receptors has yet to be determined. None of the methods employed to elucidate the subunit stoichiometry have been able to resolve this contentious issue unambiguously. The conclusions of a number of functional and biochemical studies are exactly split between a tetrameric or a pentameric structure for the NMDA receptor ion channel.

The co-expression of NR1 subunits with two subtypes of NR2 in mammalian cell lines yielded pharmacological properties that were distinct from receptors composed of NR1 subunits and one of the two subtypes of NR2 subunit suggesting that two NR2 subunits can co-assemble into the same receptor complex (Chazot *et al.*, 1994; Wafford *et al.*, 1993). In agreement with this study, the co-assembly of NR1-1a, NR2A and NR2C subunits in HEK 293 cells was shown by co-immunoprecipitation studies (Chazot *et al.*, 1994). An alternative method used by a number of groups to study subunit copy number has involved the coexpression of wild-type and mutant NMDA receptor subunits in *Xenopus* oocytes. The analysis of the resulting receptor properties was inconclusive. While the results of Premkumar and Auerbach (1997) and Laube *et al.* (1998) implied the presence of two NR2 subunits per receptor complex, (Behe *et al.*, 1995) suggested the possible co-assembly of more than two NR2 subunits. Similar studies to determine the NR1 subunit copy number were again inconclusive with either two or three NR1 subunits per receptor complex (Behe *et al.*, 1995; Premkumar and Auerbach 1997; Laube *et al.*, 1998). Finally, the co-purification of differentially epitoped-tagged NR2 subunits from transfected HEK 293 cells suggested that three NR2 subunits could co-assemble into the same receptor complex (Hawkins *et al.*, 1999). Immunoprecipitation experiments cannot, however distinguish between functional and non-functional receptors and may not therefore give results that are truly representative of native NMDA receptors.

1.4.4 Pharmacological diversity of cloned NMDA receptors

Since the cloning of the NMDA receptor subunits, a wealth of pharmacological and electrophysiological data has been accumulated. It is now evident that the receptor diversity arising from the multiple subunit genes and the alternative splicing of the NR1 gene, is paralleled by functional heterogeneity between the NMDA receptor subtypes. Thus, both the NR1 splice variant and the NR2 subtype impart different functional properties on recombinant NMDA receptors. It is the NR2 subunit however, that is the predominant factor in determining the properties of heteromeric NMDA receptors (reviewed in Zukin and Bennett, 1995; Sucher *et al.*, 1996; Yamakura and Shimoji, 1999).

Single Channel properties

The NR2 subunit subtype has significant modulatory properties on the NMDA receptors in terms of the single channel properties of recombinant NR1/NR2 receptors. The NR1/NR2A and NR1/NR2B receptors display virtually identical single channel openings to a primary conductance of 50 pS with an additional subconductance state of 40 pS. In contrast, the NR1/NR2C and NR1/NR2D receptor channels display lower primary conductance levels of 36 pS and 35 pS respectively, with subconductance states of 17 pS and 19 pS (NR1/NR2C, Stern *et al.*, 1992; NR1/NR2D, Cull-Candy *et al.*, 1998).

Ionic characteristics

Heteromeric NMDA receptors differ in both their Mg^{2+} and Zn^{2+} sensitivity and Ca^{2+} permeability. The sensitivity of heteromeric channels to Mg^{2+} block is determined by the NR2 subunit such that NR1/NR2A and NR1/NR2B are more sensitive to Mg^{2+} block than NR1/NR2C and NR1/NR2D channels (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993; Mishina *et al.*, 1993; Monyer *et al.*, 1994). The voltage-independent inhibition of heteromeric NMDA receptors by Zn^{2+} appears to be highly influenced by the NR1 splice variant and the NR2 subunit subtype. NR1/NR2A receptors are significantly more sensitive to Zn^{2+} inhibition compared to NR1/NR2B receptors, whereas both NR1/NR2A and NR1-/NR2B receptors are less sensitive to Zn^{2+} inhibition when the N1 and C2 exons are present (Williams, 1996; Traynelis *et al.*, 1998). With respect to the permeability of NMDA receptor channels to Ca^{2+} , the ratio of the Ca^{2+} influx to the total NMDA receptor-mediated current was greater for NR1-1a/NR2A receptors compared to NR1-1a/NR2C receptors suggesting that NR1-1a/NR2A ion

channels are more permeable to Ca^{2+} (Burnashev *et al.*, 1995).

L-Glutamate recognition site

The relative affinities of agonists and antagonists for heteromeric NMDA receptors composed of the different NR2 subunits have been determined primarily by recombinant expression in both *Xenopus* oocytes and HEK 293 cells. Although subunit-dependent differences in the potencies of these various compounds have been observed in both systems, differences in the relative affinities of a number of compounds depends to a certain extent on the particular expression system used. Differences in the ability of the two systems to express foreign proteins and assemble appropriate complexes may contribute to the observed differences in the pharmacological profile of recombinant NMDA receptors.

The most striking subunit-dependent difference in the pharmacology of the NMDA receptor L-glutamate site is the significant low affinity of NR1/NR2C and NR1/NR2D receptors for the agonist homoquinoloate compared to NR1/NR2A and NR1/NR2B receptors expressed in *Xenopus* oocytes (Buller *et al.*, 1994). With respect to their affinities for L-glutamate, the relative potencies determined in oocytes are NR2D>NR2C>NR2B>NR2A, whereas in HEK 293 cells they are NR2B>NR2A>NR2D>NR2C (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Laurie and Seeburg, 1994). Importantly, NR2B-containing receptors demonstrate slightly higher affinities for L-glutamate.

The NR2 subunit also controls the sensitivity of the receptor to glutamate site antagonists. In both expression systems, NR1/NR2A receptors generally display the highest affinity and NR1/NR2D the lowest affinity for L-glutamate site antagonists such as AP5, CPPene, CGS19755 and CGP 39653 (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992; Buller *et al.*, 1994; Laurie and Seeburg, 1994; Lynch *et al.*, 1994; Priestley *et al.*, 1995). However, exceptions to this trend are the compounds LY233536 and 4-(*p*-phenyl)benzoyl piperazine dicarboxylic acid (BPPD) which differ in their affinity for NR2D containing receptors. Thus, the orders of potencies for these two respective compounds are NR2B>NR2C>NR2A>NR2D and NR2B=NR2D>NR2C>NR2A.

Glycine recognition site

The NR2 subunit subtype regulates the sensitivity of the receptor to glycine site agonists and antagonists. For example, Priestly *et al.* (1995) demonstrated that NR1/NR2A

receptors displayed a 10-fold lower affinity for glycine compared to NR1/NR2B, NR1/NR2C and NR1/NR2D receptors. A similar difference in affinity has been observed for the glycine site agonists D-serine, HA-966 and (3R, 4R)-3-amino-1-hydroxy-4-methylpyrrolidin-2-one (L-687,414) (Ikeda *et al.*, 1992; Grimwood *et al.*, 1996; Buller *et al.*, 1994). Differences in the relative affinities of the heteromeric receptors for glycine-site antagonists are generally less pronounced. For example the antagonists, 7-Chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(H)quinolinone (L-701,324), L-689,560 and 7-CK, did not clearly differentiate between NMDA receptor subtypes (Grimwood *et al.*, 1995a; Priestley *et al.*, 1996). Indeed, some of the mutations in the NR1 subunit that affected the affinity of NR1/NR2 receptors for glycine agonists either did not affect or only moderately affected glycine antagonists suggesting that glycine antagonists interact with domains that are virtually insensitive to modulation by the NR2 subunit variants (Kuryatov *et al.*, 1994; Wafford *et al.*, 1995; Williams *et al.*, 1996). However, the glycine site antagonist ($\pm$)-*trans*-4-[2-(4-azidophenyl)acetyl amino]-5,7-dichloro-1,2,3,4-tetrahydro-quinoline-2-carboxylic acid (CGP 61594) has an approximate 10-fold higher affinity for NR1/NR2B receptors compared to other heteromeric subunit combinations (Honer *et al.*, 1998).

Despite the localisation of the glycine binding site to the NR1 subunit, the NR1 splice variants display equal sensitivities to glycine when expressed as homomers in *Xenopus* oocytes (Sugihara *et al.*, 1992). Furthermore, Anegawa *et al.* (1995) showed that the glycine site antagonist, [^{3} H]5,7-DCK, bound all the splice variants with equal affinity. Therefore alternative splicing of the NR1 subunit does not influence of the glycine site of the NMDA receptor.

Ion Channel Blockers

Heteromeric NMDA receptors exhibit significant subunit-dependent variations in their sensitivity to channel blockers. Notably NR1/NR2A and NR1/NR2B receptors expressed in HEK 293 cells display a 50-fold higher affinity for [^{3} H]MK801 than NR1/NR2C receptors (Chazot *et al.*, 1994; Laurie and Seeburg, 1994). In contrast, Monaghan and Larsen (1997) showed that receptors composed of either NR2 subunit displayed the same functional affinity for [^{3} H]MK801, although NR1/NR2A and NR1/NR2B receptors showed significantly quicker rates of association and dissociation, perhaps reflecting the shorter mean open times of NR1/NR2C receptors (Stern *et al.*, 1992). Therefore, it may take longer for the binding of MK801 to NR1/NR2C receptors to reach equilibrium and the apparent discrepancy between the observations of Monaghan and

Larsen (1997) and the affinities determined by other groups may have been the result of non-equilibrium conditions for NR1/NR2C receptors in radioligand binding experiments. Other non-competitive antagonists display similar pharmacological profiles to MK801, for example, N-[1-(2-thienyl)cyclohexyl]-piperidine (TCP), whereas ARL-15896 and memantine show higher affinity to NR1/NR2B receptors compared to NR2A- and NR2C-containing receptors (Porter and Greenamyre, 1995).

Regulation by Protons

The sensitivity of NMDA receptors to inhibition by protons is dependent upon both the NR2 subunit subtype and the NR1 splice variant. Protons inhibit homomeric and heteromeric channels composed of the NR1 subunit lacking the N1 insert with higher potency than channels composed of NR1 subunits containing the N1 insert (Traynelis *et al.*, 1995). In addition, the NR1/NR2C channel is exceptionally insensitive to protons compared to NR1/NR2A and NR1/NR2B receptors irrespective of the presence of the N1 exon.

Regulation by Polyamines

The four distinct mechanisms of regulation of the NMDA receptor by polyamines (described in 1.2.3) are highly dependent upon both the NR1 and NR2 subunit. Notably, NR2C and NR2D containing receptors are insensitive to all four mechanisms of regulation. Both the glycine-dependent, polyamine-induced increase in the affinity of the receptor for glycine and the voltage-dependent inhibition of NMDA receptors are displayed by NR2A- and NR2B- containing receptors (Williams *et al.*, 1994; Williams, 1995; Zhang *et al.*, 1994). In contrast, the glycine-independent stimulation of NR1/NR2 channels by low concentrations of polyamines requires the NR2B subunit and NR1 splice variants which lack the N1 exon (Lynch *et al.*, 1995; Gallagher *et al.*, 1997). In addition, the glycine-dependent inhibition by higher concentrations of polyamines due to a decrease in affinity for glutamate is only seen for NR2B containing receptors.

Non-competitive antagonism by Ifenprodil

The non-competitive antagonist ifenprodil and a number of its structurally related analogues selectively block NR2B-containing receptors by a novel activity-dependent mechanism (Gallagher *et al.*, 1996; Kew *et al.*, 1996). However, the action of ifenprodil is independent of the NR1 subunit splice variant. Other related compounds such as (1S,2S)-1-(4-Hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (CP-101,606)

and Ro 25-6981 have been developed which are also strongly selective for NR1/NR2B channels (CP 101,606, Menniti *et al.*, 1997; Ro 25-6981, Fischer *et al.*, 1997).

Cell cytotoxicity

The expression of functional NR1/NR2A or NR1/NR2B receptors in HEK 293 cells results in NMDA receptor-mediated cell death (Cik *et al.*, 1993; Anegawa *et al.*, 1995). This effect is directly related to basal activity of the expressed recombinant receptor since application of NMDA receptor antagonists prevents this cell death. Cells transfected with NR1/NR2C receptors however, were less vulnerable to excitotoxicity (Chazot *et al.*, 1994; Anegawa *et al.*, 1995). This observation is consistent with the lower conductance states of NR1/NR2C receptors (Stern *et al.*, 1992, 1994) and their lower permeability to Ca^{2+} compared to NR1/NR2A and NR1/NR2B receptors (Burnashev *et al.*, 1995).

Regulation of cloned receptors by phosphorylation

The regulation of NMDA receptor function by phosphorylation has been analysed extensively in recombinant expression systems. Initial experiments demonstrated a potentiation of NMDA-induced currents following injection of *Xenopus* oocytes with NR1 mRNA and subsequent treatment with the PKC activating agent, TPA. Indeed, since their cloning, all the NR1 splice variant homomeric receptors are known to exhibit potentiation by phorbol esters in oocytes (Durand *et al.*, 1992; Durand *et al.*, 1993; Kutsuwada *et al.*, 1992; Yamazaki *et al.*, 1992a; Mori *et al.*, 1993). The splice variants were however differentially sensitive to potentiation by PKC in that the presence of the N1 cassette conferred an increased sensitivity while the presence of the C1 cassette decreased the sensitivity (Durand *et al.*, 1992, 1993; Zheng *et al.*, 1997, 1999). The effect of the N1 exon on the potentiation of heteromeric NMDA receptors on the whole agreed with the results obtained for homomeric NR1 receptors (Kutsuwada *et al.*, 1992; Siegal *et al.*, 1994; Logan *et al.*, 1999). In addition however, the subtype of the NR2 subunit controlled the sensitivity of the receptor to potentiation by PKC. Grant *et al.*, (1998) found that PKC enhanced NR1-1a/NR2A- and NR1-1a/NR2B receptor-mediated currents, but not NR1-1a/NR2C and NR1-1a/NR2D receptor currents. Furthermore, currents mediated by NR1/NR2C and NR1/NR2D receptors lacking the C1 exon were actually suppressed by treatment with TPA. In contrast, Xiong *et al.* (1998) showed that PKC potentiated NR1/NR2A receptors but not NR1/NR2B receptors and that this potentiation was due to an increase in the probability of channel opening without changing single-channel current amplitudes or channel open times.

Thus, the regulation of NMDA receptors by PKC is dependent upon both the NR2 subtype and the NR1 splice variant. This diversity may explain the variable effects of protein kinase activators in native systems

Biochemical studies have been employed to address whether NMDA receptor subunits are actually differentially phosphorylated *in vitro* and *in vivo* and whether changes in phosphorylation accompany the PKC and PKA induced changes in channel function. The phosphorylation state of both the NR1 and NR2 subunits has been investigated by measuring incorporation of [³²P]-orthophosphate or by using phosphorylated-peptide specific antibodies. Tingley *et al.* (1993, 1997) showed that the NR1 subunit was phosphorylated in primary cultures of cortical neurons, and that the level of phosphorylation was increased after treatment with phorbol esters. The specific NR1 residues phosphorylated by PKA and PKC were subsequently mapped, revealing that three serine residues contained in the C1 exon were the major sites of phosphorylation. Serines 890 and 896 were targeted by PKC and serine 897 was targeted by PKA. Surprisingly, the residues that appear to be phosphorylated by PKA and PKC are located in a sequence that confers a decrease in sensitivity to potentiation by PKC and PKA, suggesting that the actions of PKA and PKC may not be mediated by direct phosphorylation of the NR1 subunit. Indeed, Zheng *et al.* (1999) demonstrated that PKC-mediated potentiation of NMDA receptor currents could still be observed when all the differentially spliced exons of NR1 were removed. In addition, current responses mediated by receptors containing a mutant NR1 subunit, lacking all C-terminal serine and threonine residues, were enhanced by treatment with TPA (Yamakura *et al.*, 1993).

Phosphorylation of the NR2A and NR2B subunits *in vivo* has been demonstrated, and in agreement with Tingley *et al.*, (1997) phorbol esters increased the level of phosphorylation of both NR1 and NR2 subunits (Leonard and Hell, 1997; Hall and Soderling, 1997). Omkumar *et al.* (1996) also found that NR2B subunits were phosphorylated by CaMK-II in addition to PKA and PKC, although a functional consequence of this has not been reported.

The potentiation of glutamate-activated currents by src and fyn kinases has been reconstituted in recombinant systems demonstrating a selectivity for the regulation of NR1/NR2A receptor currents (Kohr and Seeburg, 1996). Although the precise residues phosphorylated by tyrosine kinases have not been specifically mapped, tyrosine phosphorylation of NR2A and NR2B subunits has been demonstrated *in vitro* and *in vivo* (Moon *et al.*, 1994; Lau and Huganir, 1995).

Interestingly, insulin enhances NMDA receptor-mediated synaptic transmission in the CA1 region of the hippocampus (Liu *et al.*, 1995). Insulin receptors, IRS-1 and IRS-2 and their signalling components are enriched in the hippocampus and cerebral cortex (Kar *et al.*, 1993; Folli *et al.*, 1996) and are known to mediate the actions of insulin through a wide variety of kinases and phosphatases. Exposure of hippocampal slices to insulin increased the tyrosine phosphorylation of NR2A and NR2B subunits (Christie *et al.*, 1999). In recombinant expression systems, insulin enhanced NMDA receptor currents in *Xenopus* oocytes expressing NR1/NR2A, NR1/NR2B or NR1/NR2D receptors but not NR1/NR2C (Liao and Leonard, 1999). However, the effect on each receptor subtype was differentially inhibited by PTK or PKC inhibitors, suggesting that potentiation of NR1/NR2A and NR1/NR2B receptor currents was mediated by PKC while potentiation of NR1/NR2B and NR1/NR2D receptor currents was mediated by PTKs.

It is clear that the activity of protein kinases and phosphatases regulate the function of NMDA receptors bidirectionally. The challenge now lies in identifying the regulators of the specific protein kinases and phosphatases involved and how their activation results in the phosphorylation of the receptor. Clearly, the activated enzyme must come into contact with the target receptor subunit and this is likely to be a highly specific and regulated interaction. As will be discussed in section 1.9, the identification of a whole family of NMDA receptor-interacting proteins is beginning to shed some light on this highly complex regulatory system.

1.5 REGIONAL AND TEMPORAL DISTRIBUTION OF NATIVE NMDA RECEPTORS

NR1 subunits

The spatial and temporal distribution of the NMDA receptor subunits has been examined by mRNA *in situ* hybridisation and immunohistochemical analyses. In many cases, the resulting heterogenous distribution of NR2 receptor subunits explains the regional variations in NMDA receptor pharmacology that can be replicated by the respective combination of recombinant NMDA receptor subunits .

In the adult rodent, the NR1 subunit mRNA is distributed ubiquitously in the brain (Monyer *et al.*, 1994; Laurie *et al.*, 1995). The NR1 splice variants however, show distinct but overlapping regional expression profiles. The NR1-1a and NR1-2 splice variant mRNAs are detectable in most neurons of the CNS, while the NR1-3 variants are poorly

expressed in all areas (Laurie *et al.*, 1995). Interestingly the NR1-1b and NR1-4 mRNAs have a complementary expression such that NR1-1b mRNA is found most prominently in the cortex and the dentate gyrus of the hippocampus while NR1-4 mRNA is found in most other areas. Using an N1 exon specific probe, Markram *et al.* (1992) demonstrated prominent expression of N1 exon containing mRNAs in the CA3 region of the hippocampus, selected regions of the thalamic nuclei and the granule cell layers of the cerebellum. An exact correlation between the regional expression of NR1 splice variants, functional heterogeneity of native NMDA receptors, and the properties of recombinant NMDA receptor assembled from the corresponding NR1 splice variants requires further characterisation of heteromeric receptors containing different NR1 splice forms. However, the lack of potentiation by spermine of native receptors lacking the N1 exon is consistent with the requirement of the N1 exon for the potentiation of recombinant NMDA receptors by polyamines in saturating concentrations of glycine.

The NR1 subunit mRNA can be found in most neurons throughout all stages of development, although some developmental differences in the expression of NR1 mRNAs has been observed. In particular, expression of mRNAs lacking the N1 exon is high in the hippocampus at birth whereas N1 containing mRNAs are expressed at near background levels until later in development when their expression peaks at post-natal day 14 ([P14]; Paupard *et al.*, 1997). The distribution of NR1 subunits determined using splice variant-specific antibodies is mostly in close agreement with *in situ* hybridisation profile (Brose *et al.*, 1993), although additional variations in the relative distribution of the splice variants has been observed within certain defined brain structures (Standaert *et al.*, 1993; Johnson *et al.*, 1996).

NR2 subunits

The NR2 subunits have characteristic but again overlapping distribution profiles as defined by immunocytochemical and *in situ* hybridisation studies. The NR2A subunit is ubiquitously expressed in all brain regions with particularly high expression levels in the cortex, hippocampus, thalamus and cerebellum (Monyer *et al.*, 1994; Wang *et al.*, 1995; Wenzel *et al.*, 1995; Portera-Cailliau *et al.*, 1996). Immunohistochemical and *in situ* hybridisation studies were generally consistent with each other except in defining expression of NR2A mRNA in the cerebellum where Wenzel *et al.* (1995) demonstrated a high expression of NR2A subunit protein whereas NR2A mRNA was expressed at a lower level compared to other brain regions. The NR2B subunit is predominantly localised to forebrain

structures with high expression in the cortex, hippocampus and striatum. In contrast to the NR2A subunit, the NR2B subunit displays a particularly low level of expression in the cerebellum, although expression has been detected in specific cell populations within the cerebellum (Thompson *et al.*, 2000). The expression of the NR2C subunit is almost exclusively restricted to the cerebellum, although low levels are also found in the thalamus and olfactory bulb (Watanabe *et al.*, 1992; Monyer *et al.*, 1994; Wenzel *et al.*, 1995; Daggett *et al.*, 1998; Thompson *et al.*, 2000). The NR2D subunit displays an overall weak level of expression. It is predominantly expressed in thalamic regions but it is also found at low levels in the cortex, hippocampus and cerebellum (Monyer *et al.*, 1994; Wenzel *et al.*, 1995; Dunah *et al.*, 1996; Dunah *et al.*, 1998; Thompson *et al.*, 2000).

During development the expression of the NR2 subunits follow distinct temporal expression profiles. In prenatal brains the NR2B subunit displays a widespread pattern of expression whereas neither the NR2A nor the NR2C subunit is detectable (Watanabe *et al.*, 1992; Monyer *et al.*, 1994; Wang *et al.*, 1995; Portera-Cailliau *et al.*, 1996). After P12, the expression of the NR2B subunit in the cerebellum decreases dramatically and reaches undetectable levels by P22. In contrast, NR2B subunit expression is maintained in the forebrain after birth. The NR2D subunit is also expressed prenatally but it is restricted to the same regions as in the adult (Dunah *et al.*, 1996; Dunah *et al.*, 1998). NR2D expression peaks after birth at P7, then decreases approximately two-fold to adult levels. The NR2A subunit is not expressed in prenatal brains but appears at about P12 with a widespread distribution and reaches adult levels by about P22 (Wang *et al.*, 1995). Expression of the NR2C subunit occurs after P9 predominantly in the cerebellum and coincides with the decrease in NR2B expression (Wang *et al.*, 1995).

Correlations between NMDA receptor subunit expression and functional diversity

The pharmacologically distinct NMDA receptor subtypes described in section 1.2.3 colocalize in most cases with specific NR2 subunit mRNAs and protein, such that the native NMDA receptor pharmacology can be reproduced with the corresponding combination of recombinant NMDA receptor subunits expressed in *Xenopus* oocytes and heterologous cells (Buller *et al.*, 1994). Perhaps the most striking correlation between recombinant NMDA receptor properties, native receptor heterogeneity, and NR2 subunit distribution is the variation in sensitivity to channel blockers. Thus, the apparent low affinity of NR1/NR2C receptors for [³H]MK801 is paralleled by a corresponding low affinity of cerebellar receptors for [³H]MK801 and an almost exclusive distribution of

NR2C to the cerebellum. In fact, the affinity of cerebellar receptors for [³H]MK801 is more closely matched by the affinity of NR1/NR2A/NR2C receptors which is consistent with the presence of the NR2A subunit in the cerebellum in addition to the NR2C subunit (Chazot *et al.*, 1994). The characteristic pharmacological properties of the glutamate site of native cerebellar receptors are also consistent with those of NR1/NR2C receptors such that both populations of receptor bind glutamate site antagonists with low affinity and their affinities for the agonists NMDA and glutamate are relatively similar. In addition, polyamines demonstrated no modulatory effect on either native cerebellar receptors or NR1/NR2C receptors, again consistent with the restricted localization of NR2C subunits to the cerebellum.

The distribution of NR2D subunits is consistent with the distinct pharmacological properties of NMDA receptors in the medial thalamic nuclei. The relatively high affinity of receptors in this region for the glutamate site antagonists, LY-233536 and BPPD may be explained by the restricted distribution of NR2D subunits to this region, and the corresponding high affinity of NR1/NR2D receptors for these antagonists.

The distribution of the remaining two classes of pharmacologically distinct native NMDA receptors in the forebrain can be closely correlated with the localization of NR2A and NR2B subunit mRNAs (Buller *et al.*, 1994). Both NR2A- and NR2B-containing cloned receptors display relatively high affinity binding sites for ion channel blockers and L-glutamate site antagonists accounting for the pharmacological properties of native forebrain receptors compared to cerebellar receptors. In addition, the differences between the antagonist and agonist selectivity of cloned NR2A- and NR2B-containing receptors explains the antagonist- and agonist-preferring properties of native forebrain receptors. Only those regions in the forebrain that contain NR2A subunits display high affinity [³H]CPP binding sites, whereas those regions containing NR2B subunits display low relative affinities for glutamate antagonists and relatively high affinities for agonists. The high sensitivity of the striatum to the potentiation of [³H]MK801 binding by polyamines can also be explained by the enrichment of the striatum with NR2B subunits and the corresponding NR2B subunit-dependence of spermine potentiation of heteromeric recombinant receptors.

Although in most cases the pharmacological properties of native NMDA receptor can be explained by the distribution of NR2 subunit subtypes, the properties of cloned NMDA receptors are not always representative of their native counterparts. For example, the affinities of cloned NR1/NR2 receptors for glycine is significantly reduced for all

subunit combinations with the exception of NR1/NR2C compared to native NMDA receptors (Laurie and Seeburg, 1994). Therefore, additional factors may contribute to the functional heterogeneity of native NMDA receptors.

1.6 QUATERNARY STRUCTURE OF NATIVE NMDA RECEPTORS

As described in section 1.5, the characterisation of the pharmacological and electrophysiological properties of cloned NMDA receptors explains in most cases the functional heterogeneity displayed by native NMDA receptors. However, it is important that the NMDA receptor subunit assemblies generated in recombinant expression systems are actually representative of native receptors. For example, even though the NR1 subunit is considered to be an obligatory subunit for the expression of functionally active cloned NMDA receptors and can be co-immunoprecipitated with NR2 subunits in mammalian cell lines, proof that indeed the NR1 subunit is mandatory *in vivo* requires demonstration of the co-assembly of NR1 with NR2 subunits in native receptor complexes. The ubiquitous expression of the NR1 subunit mRNA and protein in mammalian brains is of course consistent with this requirement of the NR1 subunit. Furthermore, since the L-glutamate and glycine recognition sites have been mapped to the NR2 and NR1 subunits respectively, it is highly likely that native NMDA receptors are composed of NR1 and NR2 subunits.

It is also essential for the accurate interpretation of investigations performed on cloned heteromeric receptors, to know whether the NR1 splice variants and NR2 subunit subtypes that display overlapping expression profiles *in vivo* can co-assemble into the same native NMDA receptor complex. As discussed in section 1.4.3, the subunit stoichiometry of cloned NMDA receptors indicates co-assembly of at least two NR1 and two NR2 subunits. It is therefore possible that the assembly of native NMDA receptor subunits results in the formation of a receptor complex comprising two different NR1 splice variants and two or three NR2 subunit subtypes. Since the majority of the studies performed on cloned NMDA receptors have involved the co-expression of an NR1 subunit with a single subtype of NR2 subunit, it is important to know what proportion of native receptors contain specific combinations of NR2 subunit.

To investigate the co-assembly of NMDA receptor subunit subtypes, immunoprecipitation and immunopurification studies have been employed. As expected these studies revealed that native NMDA receptors are heteromeric complexes composed of NR1 and NR2 subunits. Furthermore, native NMDA receptors can contain at least two

different NR1 splice variants and at least two subtypes of NR2 subunit. Blahos and Wenthold, (1996) demonstrated the co-association of NR1 subunits containing C2 and C2' exons, neither of which can appear in the same NR1 subunit. They also found that little NR2A was co-immunoprecipitated with NR2B subunits suggesting the preferential co-assembly of NR1 subunits with a single type of NR2 subunit. In a similar study, Chazot and Stephenson (1997a) showed that the majority of receptors exist as either NR1/NR2A or NR1/NR2B receptors although a minority of NR2A and NR2B subunits could co-assemble with NR1 subunits in the same native receptor complex. The relative proportions of the native NMDA receptors containing a specific NR2 subunit remains a contentious issue. For example, Luo *et al.* (1997) suggest that the majority of native NMDA receptors in the forebrain are in fact represented by the NR1/NR2A/NR2B combination of subunits and that the NR1/NR2A and NR1/NR2B subunit combinations account for only a small proportion of native heteromeric receptor complexes. The co-assembly of two different NR2 subunits into the same receptor complex has also been shown in recombinant studies where the cotransfection of NR1, NR2A and NR2C subunits in HEK 293 cells resulted in the expression of receptors with distinct pharmacological properties compared to NR1/NR2A or NR1/NR2C receptor combinations (Chazot *et al.*, 1994). Even though the properties of cerebellar receptors were more closely matched by the properties of cloned NR1/NR2A/NR2C, Didier *et al.* (1995) found that in the postnatal mouse cerebellum the NR2C subunit was not co-assembled with NR2A or NR2B. Additional immunoprecipitation studies have shown that NR2D subunits co-assemble with NR1 and NR2A subunits in some brain regions but only with NR1 subunits in other brain regions. In summary, it is clear that different NR1 splice variants and different NR2 subtypes of NR2 subunit are able to co-assemble into the same receptor complex but that the co-assembly of more than one NR2 subtype into the same complex appears to vary between different brain regions (Dunah *et al.*, 1998). These investigations suggest that a simple correlation between NMDA receptor subunit distribution, properties of heteromeric receptors and the properties of native receptors may be complicated by the preferential co-assembly of NMDA receptor subunit subtypes despite their co-localization.

With respect to the stoichiometry of native NMDA receptors, functional studies by Patneau and Mayer (1990) and Beneveniste and Mayer (1991) demonstrate that agonist dose-response curves are consistent with the binding of at least two molecules of glutamate and two molecules of glycine per receptor complex. Since the glutamate and glycine binding sites have been mapped to the NR2 and NR1 subunits respectively, this would

imply that at least two NR1 subunits co-assemble with at least two NR2 subunits. The molecular size of the native NMDA receptor determined by native polyacrylamide gel electrophoresis is in the range of 603-850 kDa, suggesting the assembly of four to six subunits (Brose *et al.*, 1993; Chazot *et al.*, 1994; Blahos and Wenthold, 1996). Thus, as with recombinant NMDA receptors the precise copy number of native NMDA receptor subunits co-assembled to form a functional ion channel remains to be determined.

1.7 FUNCTIONS OF NMDA RECEPTORS

The function of NMDA receptors in synaptic transmission and synaptic plasticity

Glutamate receptors constitute the major excitatory transmitter system in the CNS of the mammalian brain. The importance of NMDA receptor-mediated events to the general physiology of the brain was demonstrated by the perinatal death of genetically altered mice lacking either the NR1 or NR2B subunit (Kutsuwada *et al.*, 1996; Forrest *et al.*, 1994). In wild-type mice both AMPA/kainate and NMDA receptor antagonists severely disrupt synaptic events, although NMDA receptor antagonists reduce a later component of synaptic currents due to the longer lasting activation of NMDA receptors. A number of significant differences in the properties of NMDA and non-NMDA receptors are critical to their role in the CNS. With the use of selective antagonists, AMPA and kainate receptors were shown to be involved in the fast synaptic excitation in the brain and spinal chord. NMDA receptors however, due to their voltage-dependence and Ca^{2+} permeability are thought to play a more complex role in the regulation of synaptic excitability. The permeability of the NMDA receptor to Ca^{2+} enables the NMDA receptor to participate in numerous downstream signalling pathways that can ultimately lead to profound changes in the properties of both the pre-synaptic and post-synaptic neurons. The NMDA receptor is also the only ligand-gated ion channel whose probability of opening depends strongly upon the voltage across the membrane. It is these two properties that have implicated the NMDA receptor in such diverse processes as synaptogenesis, synaptic plasticity and neurotoxicity. Notably, the ability of the NMDA receptor to detect two coincident inputs, namely the release of neurotransmitter from the pre-synaptic neuron and depolarization of the post-synaptic cell, permits the receptor to operate as a Hebbian molecule (Hebb, 1954). Hebb first postulated that the molecular basis of learning and memory was likely to be mediated by the strengthening of a synapse when both the pre- and post-synaptic neurons were simultaneously active. Detection of this simultaneous activity required the presence of a

theoretical molecule, a Hebbian molecule, capable of detecting two co-incidental inputs.

The major forms of NMDA receptor-mediated changes in synaptic transmission are the long term changes in the efficiency of transmission at glutamatergic synapses following either low-frequency or high frequency stimulation of afferent pathways. Bliss and Lømo (1973) demonstrated long-term potentiation (LTP) of synaptic transmission in the dentate area of the rabbit following high frequency stimulation of the perforant pathway. Conversely, a train of low-frequency stimulation produces a reduction in synaptic efficacy known as long term depression (LTD). The mechanisms thought to underlie the modulation of synaptic transmission include changes in the properties of post-synaptic glutamate receptors, changes in receptor density, structural alterations in the dendritic spine and the generation of retrograde signals to the pre-synaptic neuron (reviewed in Malenka, 1994; Sweatt, 1999; Elgersma and Silva, 1999; Soderling and Derkach, 2000). These cellular responses are thought to be triggered largely by the activation of NMDA receptors during afferent stimulation followed by the entry of Ca^{2+} into the post-synaptic cell. Indeed, genetically altered mice completely lacking either the NR2A, NR2C or NR2D subunit were all viable but displayed varying deficiencies in hippocampal plasticity (Ikeda *et al.*, 1995; Sakimura *et al.*, 1995; Ebrailidze *et al.*, 1996; Kadotani *et al.*, 1996). The signalling pathways downstream of NMDA receptor activation have therefore been the focus of many neurochemists.

A multitude of Ca^{2+} -dependent signalling pathways have been implicated in the induction and maintenance of LTP and LTD. In particular, the identification of CaMK-II as a necessary factor for the induction of LTP was a critical discovery (Malenka *et al.*, 1989; Malinow *et al.*, 1989). Several studies have shown that activated CaMK-II is translocated into the vicinity of the post-synaptic membrane and that one of its substrates is the NMDA receptor (Strack and Colbran, 1998). Although CaMK-II has been shown to phosphorylate the NR2B subunit, a functional consequence of this has not been reported. A variety of other substrates for CaMK-II however, are known to participate in many of the very pathways responsible for the changes in the excitability of the synapse (reviewed in Soderling, 2000). For example, CaMK-II phosphorylates AMPA receptors during LTP resulting in an increased ion channel conductance (Barria *et al.*, 1997; Derkach *et al.*, 1999). Other substrates for CaMK-II include the transcription factor, CREB (cAMP response element binding protein), which may be involved in the enhanced transcription that is known to accompany some forms of synaptic plasticity. In addition to CaMK-II, the role of other kinases such as src, fyn, PKC and PKA in the enhancement of AMPA receptor

currents during LTP has been postulated (reviewed in Soderling and Derkach, 2000). Conversely, dephosphorylation of AMPA receptors is thought to accompany NMDA receptor-dependent LTD. (Lee *et al.*, 1998). Ca^{2+} influx through the NMDA receptor ion channel also stimulates the mitogen-activated protein kinase (MAPK) pathway, which is subsequently important for the induction of LTP via the activation of gene transcription (Bading and Greenberg, 1991; Xia *et al.*, 1996; English and Sweatt, 1996, 1997). The activity of neuronal nitric oxide synthase (nNOS), located at post-synaptic sites, is also regulated by Ca^{2+} and the subsequent synthesis of nitric oxide (NO) may mediate retrograde signalling to the pre-synaptic neuron during some forms of LTP (reviewed in Brenman and Brecht, 1997).

In addition to the activation of PKA, PKC, CaMK-II and tyrosine kinases in LTP following NMDA receptor activation, the NMDA receptors themselves are known to be regulated during the induction of LTP resulting in an increase in NMDA receptor currents. It is possible therefore that these activated kinases regulate the activity of NMDA receptors during LTP. Indeed, an increase in the phosphorylation of the NR2B subunit during LTP has been demonstrated *in vivo* (Rostas *et al.*, 1996; Rosenblum *et al.*, 1996).

A common theme to most of the changes in the electrophysiological and morphological properties of neurons that occur during the long term modulation of the efficiency of synaptic transmission is the complex nature of the signalling pathways responsible for these changes. Undoubtedly the localization of signalling molecules to the vicinity of the NMDA receptor-mediated Ca^{2+} influx and the regulation of the association of these factors with their targets is critical for the function of NMDA receptors in synaptic plasticity.

NMDA receptors and disease

The permeability of NMDA receptors to Ca^{2+} has implicated NMDA receptors in the excitotoxicity that is thought to be responsible for the neuronal cell death that accompanies stroke. The NMDA receptor was therefore a promising target for therapeutic intervention. Antagonists targeted to the glycine site, L-glutamate site and ion channel pore of the receptor have however been unsuccessful in the clinic (reviewed in Chazot and Hawkins, 1999; Keyser *et al.*, 1999). The NMDA receptor has also been a target for the treatment of pain, epilepsy, depression, Huntington's disease, schizophrenia and Parkinson's disease. Although to date, most NMDA receptor selective compounds have not reached the clinic, the growing understanding of NMDA receptor function and dysfunction

suggests that the receptor may yet prove to be a lucrative target for drug therapy.

1.8 ULTRASTRUCTURAL LOCALIZATION OF NMDA RECEPTORS TO THE POST-SYNAPTIC DENSITY

The post-synaptic localization of NMDA receptors

Immunofluorescent and immunogold labelling studies have shown that both NMDA and AMPA receptors are highly concentrated and clustered in the post-synaptic membranes of excitatory neurons (reviewed in Nusser, 2000). In all high resolution images of post-synaptic neurons, most glutamate receptors are in close apposition to a thick protein band that lies just beneath the post-synaptic membrane, known as the post-synaptic density (PSD). At higher resolutions the precise position of glutamate receptors within a single post-synaptic membrane can be observed. AMPA receptor immunoparticles exhibit either a uniform distribution (Nusser *et al.*, 1994) within the synaptic specialization or a more concentrated distribution at the periphery (Somogyi *et al.*, 1998). NMDA receptors are either concentrated at the centre (Somogyi *et al.*, 1998) of the synapse or are evenly distributed, whereas metabotropic receptors have a peripheral distribution (Baude *et al.*, 1993; Nusser *et al.*, 1994). Importantly, AMPA and NMDA receptors can colocalize in the same post-synaptic membrane (Kharazia *et al.*, 1999).

Using EM immunocytochemistry, AMPA receptors have also been detected intracellularly (Molnar *et al.*, 1993) in association with membranous cellular structures such as the Golgi apparatus and the endoplasmic reticulum (ER) although a precise quantification of the ratio of cell surface receptors to intracellular receptors is still lacking. Initial studies investigating the subcellular distribution of NMDA receptors suggested that a large proportion of NMDA receptors were present intracellularly. In contrast to these studies however, more recent methods of analysis have suggested that the majority of NMDA receptors are expressed on the surface of neurons, at synaptic junctions (Fritschy *et al.*, 1998). Interestingly, both AMPA and NMDA receptors also localize extra-synaptically to varying degrees (Molnar *et al.*, 1993; Baude *et al.*, 1994; Li *et al.*, 1998; Tovar and Westbrook, 1999). Despite this heterogeneity in the precise localisation of L-glutamate receptors, the majority of glutamatergic synapses exhibit a concentration of AMPA and NMDA receptors in the post-synaptic membrane with an abrupt decrease in their density at the edge of the PSD.

The PSDs of excitatory neurons were first detected by the electron microscope

in the 1950s as electron-dense specializations of post-synaptic membranes at points of contact between cortical neurons. PSDs appear as semicircular bands that follow the contour of the post-synaptic spine head and are found directly opposite the specialised active zone of the pre-synaptic terminal (reviewed in Ziff, 1997). They measure about 400 nm long and 40 nm wide and are composed of both filamentous and particulate components. Freeze fracture studies have identified filamentous structures within the PSD and extending into the PSD from the submembrane cytoskeleton.

PSDs can be isolated by detergent extraction under specific conditions that also retain the post-synaptic membrane allowing the identification of co-associated membrane proteins (Gurd *et al.*, 1983). Major membrane proteins identified in these isolated PSD preparations include the NMDA receptor NR1, NR2A and NR2B subunits, AMPA ionotropic receptors and metabotropic glutamate receptors (Moon *et al.*, 1994; Clark *et al.*, 1998). Interestingly, the detergent extraction of NR1 and NR2 subunits was significantly more efficient from transfected HEK 293 cells compared to forebrain membranes, suggesting the association of native NMDA receptors with insoluble cellular structures (Chazot and Stephenson, 1997b). Numerous biochemical studies have employed detergent extraction of PSDs, differential centrifugation and fractionation to isolate individual protein components of the PSD. These systematic studies have identified over 30 PSD constituents which include structural proteins such as actin and α -actinin-2, and regulatory proteins such as α CaMK-II, protein kinase A and fyn tyrosine kinase (reviewed in Ziff, 1997; Walikonis *et al.*, 2000).

The search for NMDA receptor-interacting proteins

It had been fully established from the work performed in the 1980s and early 1990s that the NMDA receptor participated in the regulation of synaptic transmission. It was clear that the Ca^{2+} permeability of the NMDA receptor ion channel was responsible for many of the activity-dependent synaptic changes that could be observed in excitatory synapses in the CNS. In addition, the NMDA receptor itself had been identified as a highly regulated ion channel and that the control of this regulated state was likely to be crucial in determining the excitability of the post-synaptic neuron. Many of the proteins involved in the signalling mechanisms responsible for the Ca^{2+} dependent changes in synaptic efficacy such as protein kinases, had been identified and furthermore localized to the PSD. These results suggested that the association of NMDA receptors with proteins in the PSD was potentially responsible for the regulation of NMDA receptor function and for the NMDA receptor-

mediated changes in synaptic activity. The biochemical and ultrastructural studies of the early 1990s also indicated that the PSD provided a structural matrix which clustered ion channels in the post-synaptic membrane. However, direct biochemical evidence for the association of NMDA receptors and indeed other glutamate receptors with either the regulatory or cytoskeletal proteins present in the PSD was necessary to dissect the pathways responsible for the control of NMDA receptor function, localization and cell surface expression. In addition, the mechanisms responsible for the complex distribution of different classes of ligand-gated ion channels to separate post-synaptic sites within the same neuron were little understood. The targeting of these ion channels, including NMDA receptors, was also likely to occur via interactions with other neuronal proteins.

The importance of receptor-associated proteins in neurotransmission had already been shown at the neuromuscular junction where the prototypical nAChR-interacting protein, rapsyn, in addition to other downstream interacting proteins, was crucial for the spatial compartmentalization and cytoskeletal interactions of nAChRs. Functional impairment of these receptor-associated proteins resulted in abnormal neurotransmission in the muscle (reviewed in Hall and Sanes, 1993). The association of the microtubule-associated protein gephyrin, was also known to be essential for the synaptic localisation of glycine receptors (reviewed in Kuhse *et al.*, 1995). With the development of the yeast-two-hybrid system (Fields and Song, 1989), the identification of NMDA receptor-associated proteins soon followed and the understanding of the biochemical pathways involved in the regulation and structural formation of glutamatergic synapses was rapidly accelerated.

The large intracellular C-terminus of the NMDA receptor NR2 subunit was a promising target for possible interacting synaptic proteins. Kornau *et al.* (1995) carried out a yeast two-hybrid screen of a rat brain cDNA library using the NR2B C-terminus as bait. Thus, the first NMDA receptor-interacting protein identified using this system was PSD-95 (alternative nomenclature synapse-associated protein (SAP)-90). In fact, PSD-95 had already been identified as a major protein in the PSD and was subsequently cloned (Cho *et al.*, 1992; Kistner *et al.*, 1993). The study by Kornau *et al.* (1995) was however, the first direct demonstration of an interaction between the NMDA receptor and PSD-95. This initial discovery was followed by the identification of a whole family of NMDA receptor-interacting proteins and in turn, a collection of downstream interacting proteins. The search for the biochemical entities responsible for the NMDA receptor-activated signalling pathways has culminated in the isolation of an NMDA multiprotein complex comprising 77 distinct proteins (Husi *et al.*, 2000). A large number of proteins associated with the other

receptors of the CNS, such as AMPA and GABA receptors, have also been cloned and characterised. In many cases, proteins associated with different ion channel receptors display very similar structures and functions. For example, a number of these proteins are now known to be involved in the clustering, targeting and cytoskeletal attachment of ion channels at synaptic sites (reviewed in Kirsch *et al.*, 1996; Sheng and Wyszynski *et al.*, 1997; Ponting *et al.*, 1997; Nagano *et al.*, 1998; Kim and Huganir, 1999; Garner *et al.*, 2000). Indeed, the regulation of synaptic localization and surface expression of ion channels by receptor-associated proteins may be a crucial mechanism responsible for the changes in the efficiency of synaptic transmission. Receptor-associated proteins are also known to regulate the activity of their target ion channel, either by a direct interaction or via the recruitment of regulatory molecules such as protein kinases. Furthermore, receptor-associated proteins are known to link various forms of synaptic activity to the neuronal signalling pathways that may be responsible for changes in synaptic plasticity and for developmental changes in neuronal structure.

1.9 THE PSD-95 FAMILY OF NMDA RECEPTOR-INTERACTING PROTEINS

1.9.1 Identification and cloning of the PSD-95 family

PSD-95 was cloned by screening a rat brain expression library with rabbit antisera raised against PSD proteins (Kistner *et al.*, 1993). Antibodies affinity-purified from this antisera using a glutathione S-transferase (GST) fusion protein encoded by the isolated clone were immunoreactive with a 90 kDa protein present only in brain membrane preparations. This protein was therefore termed synapse-associated protein 90 (SAP-90). The same protein was also cloned by another group and was identified as a 95 kDa protein present in rat brain PSDs, hence the name PSD-95 (Cho *et al.*, 1992). The nucleotide sequence of PSD-95 contains an open reading frame for a protein with 724 amino acid residues and a calculated $M_r = 80,396$. Analysis of the genomic DNA encoding human PSD-95 revealed that the gene consists of 22 exons, three of which undergo alternative splicing (Stathakis *et al.*, 1999). Analysis of the amino acid sequence by hydrophobicity plot indicated that PSD-95 lacked both membrane spanning domains and hydrophobic sequences that could insert into the plasma membrane. The protein shares 54 % amino acid identity to the *Drosophila discs-large (dlg)*-A gene product (Woods and Bryant, 1991) over

the entire length of PSD-95. The regions of homology shared by *dlg* and PSD-95 are clustered into three domains. The first domain contains three novel 90-amino acid repeats and shows similarity to a variety of cytoskeletal proteins. The second domain is a *src* homology 3 (SH3) region and the third domain is similar to the yeast guanylate kinase (GK). The 90 amino acid repeats were originally termed GLGF domains due to the presence of the relatively conserved amino acid element, Gly-Leu-Gly-Phe. However, the identification of these domains in *dlg* and in two components of the zonula occludens (ZO-1 and ZO-2), led to their renaming as PDZ (PSD-95, Dlg and ZO-1) domains (Kennedy, 1995). SH3 domains are found in the *src* family of non-receptor tyrosine kinases, in several membrane cytoskeletal proteins and membrane-associated signal transduction proteins. They are thought to mediate the clustering of cytoplasmic signal-transducing proteins at the plasma membrane and also to regulate their activity. The guanylate kinase domain appears to lack enzyme activity, although it has been shown to bind guanosine monophosphate (GMP) with micromolar affinity (Kistner *et al.*, 1995).

Following the initial characterisation of PSD-95, a number of additional proteins displaying the same characteristic profile of protein domains were identified as NMDA receptor-interacting proteins. SAP-97 was originally identified as the human homologue of *dlg* (Lue *et al.*, 1994) and exhibits a strong overall sequence similarity to PSD-95 (Muller *et al.*, 1995). Furthermore, SAP-97 interacts with NR2A and NR2B subunits in the yeast two-hybrid system (Neithammer *et al.*, 1996). PSD-93/Chapsyn-110 (channel-associated protein of synapses-110) was cloned as an NMDA receptor-interacting protein (Brenman *et al.*, 1996a; Kim *et al.*, 1996) and displays 70-80 % amino acid identity to PSD-95 and SAP-97. The fourth member of this family, SAP102, was identified by screening a rat cerebellar cDNA library using the PDZ1 domain of PSD-95. SAP102 shares 70 % amino acid identity with PSD-95 (Muller *et al.*, 1996). The presence in all of these proteins of three PDZ domains, an SH3 domain and a GK domain defines these proteins as the PSD-95 family of the MAGUK (membrane-associated guanylate kinase) superfamily.

The association of this subclass of MAGUK proteins with ion channels of the CNS is not restricted to NMDA receptors. For example, an association of PSD-95 and SAP-97 with specific subtypes of K⁺ channel has been demonstrated (Kim *et al.*, 1995; Nehring *et al.*, 2000). In addition, PSD-95, SAP102, and SAP97 from brain extracts co-immunoprecipitate native GluR6 subunits, while PSD-95 and SAP102 but not SAP97 co-immunoprecipitate the KA2 subunit (Garcia *et al.*, 1998). In addition, an association of SAP97 with the AMPA receptor subunit, GluR1, was found (Colledge *et al.*, 2000)

1.9.2 Structural domains involved in PSD-95/NMDA receptor protein-protein interactions

The initial study by Kornau *et al.* (1995) that identified PSD-95 as an NMDA receptor-interacting protein also mapped the regions of PSD-95 and NR2B responsible for the interaction. Yeast reporter gene activation studies demonstrated the requirement of the second PDZ domain of PSD-95 and the NR2B C-terminal seven amino acids for the interaction. A selective interaction with PDZ2 was also observed for NR2A, NR2C, NR2D and the NR1-3/4 splice variants. Subsequently however, it has been shown that in fact all three PDZ domains of PSD-95 can bind NMDA NR2 subunits but with differing affinity. The second PDZ domain demonstrates the highest affinity for NR2B and K⁺ channel C-termini ($K_D < 10$ nM), whereas PDZ1 has an intermediate affinity ($K_D \sim 30$ nM), and PDZ3 has a relatively low affinity ($K_D > 1$ μ M) (Muller *et al.*, 1996). Examination of the C-termini of NMDA receptor subunits and certain K⁺ channels revealed a common amino acid sequence motif termed the tSXV motif (X representing any amino acid, and tS representing T and S compatibility at this residue). Mutational analysis of this motif identified the 0 and -2 residue positions as critical for mediating an interaction with the PDZ2 domain of PSD-95 (Kim *et al.*, 1995; Bassand *et al.*, 1999). In the study by Bassand *et al.* (1999) however, an interaction between the NR1-3/4 splice variants was not observed even though these NR1 proteins have a consensus tSXV C-terminal motif.

PDZ domains have now been identified in over 70 different proteins and are well known domains mediating protein-protein interactions (reviewed in Ponting *et al.*, 1997). They are often present in multiple copies ranging from a single copy up to 9 PDZ repeats. No residues are absolutely conserved for all known PDZ domains except for the second glycine in the common GLGF amino acid element. Thus, different PDZ domains demonstrate both differing affinities for target peptides, as illustrated by the interaction of PSD-95 PDZ domains with the NR2 subunit, and demonstrate different specificities of binding (Songyang *et al.*, 1997).

The elucidation of the crystal structure of PDZ domains has identified important 3-dimensional features of both the protein structure and sites of interaction with target sequences (Doyle *et al.*, 1996; Cabral *et al.*, 1996; Tochio *et al.*, 2000). The PDZ domain is a compact globular structure containing six β strands and two α helices as shown by the schematic representation of the PDZ2 domain of PSD-95 in Figure 1.6. The peptide-complexed structure of PSD-95-PDZ3 shows a nine-residue C-terminal peptide bound in

the surface groove formed between $\beta 2$ and $\alpha 2$ (Doyle *et al.*, 1996). The C-terminal four amino acids of the peptide are highly ordered. The threonine residue at position -2 hydrogen bonds with a well conserved histidine residue located in the $\alpha 2$ helix, while the terminal valine residue binds via its free carboxylate group to a hydrophobic pocket that is formed partly by the conserved GLGF amino acid element. These specific sites of interaction are therefore in agreement with the apparent requirement for threonine and valine at amino acid positions -2 and 0 respectively for the interaction of PSD-95 with Kv1.4 channel subunits (Kim *et al.*, 1995).

1.9.3 Regional distribution of the PSD-95 family of proteins

A prerequisite for a physiological interaction of the NMDA receptor subunits with the PDZ domains of the PSD-95 family of MAGUKs is that they share regional, cellular and subcellular localisation. In general, the distribution of PSD-95, SAP102 and PSD-93 in the brain is similar and the localisation of their mRNAs and protein overlaps with the distribution of NMDA receptors (Kornau *et al.*, 1995; Bassand *et al.*, 1999). Of the PSD-95 family, SAP97 and PSD-93 are the only members that are not brain specific. PSD-93 is predominantly distributed to neuronal cell populations, although expression has been detected in specific non-neuronal cells (Brenman *et al.*, 1996a). SAP97, in addition to a widespread distribution in the CNS, is also found in a variety of epithelial cells and in B-lymphocytes (Lue *et al.*, 1994; Muller *et al.*, 1995). Some regional variation in the expression of the PSD-95 family of MAGUKs in the CNS exists. For example, PSD-95 and PSD-93 are abundant in the cerebral cortex, hippocampus, and in subcortical regions, but are present at lower levels in cerebellum (Kim *et al.*, 1996). In contrast, SAP102 is found at high levels in all these regions including the cerebellum (Muller *et al.*, 1996). The distribution of SAP97 in the CNS follows distinct regional variations. Its expression is highest in the cerebellum and is relatively low in the hippocampus and cerebral cortex (Kim *et al.*, 1996; Bassand *et al.*, 1999).

Interestingly, a marked difference in the distribution of the PSD-95 family of proteins is observed in the cerebellum where in Purkinje neurons, PSD-93 is the only MAGUK present (Kim *et al.*, 1996; Brenman *et al.*, 1998; Bassand *et al.*, 1999). Conversely, in the granule cell layer PSD-95, SAP102, and SAP97 are all co-localised, whereas PSD-93 is absent.

The subcellular distribution of PSD-95, SAP102 and PSD-93 is consistent with

an interaction with NMDA receptors at the post-synaptic density. PSD-95 was originally identified as a major component of the post-synaptic density and distributes primarily to post-synaptic sites by immunogold labelling and by green fluorescent protein (GFP) tagging (Cho *et al.*, 1992; Hunt *et al.*, 1996). However, a minority of PSD-95 found in the CNS is localised pre-synaptically in cerebellar basket cells (Kistner *et al.*, 1993; Hunt *et al.*, 1996) suggesting a possible association with pre-synaptic membrane proteins such as specific subtypes of K⁺ channels which are found predominantly in pre-synaptic nerve terminals (Sheng *et al.*, 1992). Biochemical analysis of crude synaptosomal membrane fractions has demonstrated the co-purification of PSD-95, PSD-93 and SAP102 with post-synaptic densities, and a resistance of these proteins to detergent extraction (Kim *et al.*, 1996; Muller *et al.*, 1996). A direct association of PSD-95, PSD-93 and SAP102 with NMDA receptors *in vivo* has been difficult to demonstrate due to this resistance of PSD-95-like proteins to detergent extraction. However, the co-immunoprecipitation of NMDA receptors and associated MAGUK proteins from solubilised rat brain membranes has been demonstrated (Sans *et al.*, 2000; Husi *et al.*, 2000).

In contrast to the localization of PSD-95, PSD-93 and SAP102 primarily to post-synaptic neurons, SAP97 is found predominantly in the pre-synaptic nerve termini of excitatory synapses in the hippocampus and cerebellum, and in and along unmyelinated axons in other brain regions (Muller *et al.*, 1995; Kim *et al.*, 1996). However, a somatodendritic distribution of SAP97 in some regions of the hippocampus is suggestive of a post-synaptic distribution in selected neurons (Bassand *et al.*, 1999). SAP97 does not copurify with PSD-95 in post-synaptic density preparations, and is not resistant to detergent extraction consistent with a pre-synaptic localisation (Kim *et al.*, 1996). The identification of NMDA receptors in pre-synaptic compartments (Conti *et al.*, 1997) suggests a possible association of SAP97 with pre-synaptic NMDA receptors or, alternatively, with other unidentified membrane proteins.

The expression of the PSD-95 family of MAGUK proteins also varies developmentally in the rat hippocampus (Sans *et al.*, 2000). SAP102 appears relatively early at P2 when PSD-95 and PSD-93 are not expressed. By P10, SAP102 expression has peaked, while PSD-95 and PSD-93 are still weakly expressed. After 6 months, PSD-95 and PSD-93 are at maximum expression levels whereas SAP102 expression has declined slightly to adult levels.

1.10 NON-PSD-95-LIKE NMDA RECEPTOR-INTERACTING PROTEINS

Although the C-terminal regions of the NR2 subunits are important for the interaction of NMDA receptors with the PDZ-containing PSD-95 family of MAGUK proteins, the association of NMDA receptors with other regulatory and cytoskeletal proteins occurs via different protein domains located in both the NR1 and NR2 subunits. A summary of the 'first generation' of NMDA receptor-interacting proteins, *i.e.* those interacting directly with the NMDA receptor, is shown in Figure 1.7.

The first direct evidence of an association of NMDA receptors with cytoskeletal proteins was presented by Wyszynski *et al.* (1997). Using the yeast two-hybrid system and *in vitro* binding assays, α -actinin 2, a member of the spectrin/dystrophin family of actin binding proteins, was shown to bind NR1 and NR2B subunits. α -Actinin 2 shows punctate co-localisation with NMDA receptors in the PSDs of glutamatergic synapses (Wyszynski *et al.*, 1998). It is proposed to function as a bridging protein between NMDA receptors and actin at post-synaptic sites. The cytoskeletal proteins spectrin and soluble forms of tubulin also interact directly with the NMDA receptor. Spectrin, a protein that links membrane proteins to the actin cytoskeleton interacts with the C-terminal domains of NR1-1a, NR2A and NR2B (Wechsler and Teichberg, 1998), whereas the NR1-1a and NR2B C-termini bind tubulin (Rossum *et al.*, 1999). The NR1 subunit also interacts, in a splice variant-dependent manner, with another cytoskeletal protein, the light neurofilament subunit (NF-L; Ehlers *et al.*, 1998). NR1 splice variants lacking the C1 cassette do not associate with NF-L.

A variety of signalling proteins also associate with the NMDA receptor including calmodulin, CaMK-II, phospholipase C, and yotiao (Wyszynski *et al.*, 1997; Strack and Colbran, 1998; Lin *et al.*, 1998; Gurd and Bissoon, 1997).

1.11 PSD-95-INTERACTING PROTEINS

Once the association of the PSD-95 family of proteins with NMDA receptors had been verified, various regions of PSD-95 were used as bait in further yeast two-hybrid screens of rat brain cDNA libraries to identify downstream interacting proteins. The regions of PSD-95 that mediate the assembly of these multiprotein complexes include all three PDZ domains, the SH3 domain and the GK domain. A list of these 'second generation' interacting proteins and their sites of interaction is shown in Figure 1.8. A summary of yet more downstream interacting proteins that associate with these PSD-95 binding partners

is shown in Figure 1.9. The functional significance of some of these additional downstream proteins is described in section 1.12.

1.12 FUNCTIONS OF THE PSD-95 FAMILY OF NMDA RECEPTOR-INTERACTING PROTEINS

1.12.1 Ion channel clustering

As described in section 1.8, NMDA, mGluR and AMPA receptors are highly localised to the PSD and are undoubtedly clustered into these receptor-rich domains by their respective associated proteins. The clustering of ion channels at synaptic sites is important for concentrating current flux across a small area of the membrane and for maximizing responses to local neurotransmitter release. Furthermore, changes in the distribution and density of glutamate receptors may play a role in long term synaptic plasticity and information storage in the brain.

PSD-95 has been directly implicated in the synaptic clustering of its associated ion channels. This has been dramatically demonstrated by the co-expression of PSD-95 with NMDA receptors, K⁺ channels and kainate receptors in heterologous cells (Kim *et al.*, 1995; Kim *et al.*, 1996; Horio *et al.*, 1997; Kim and Sheng, 1996; Garcia *et al.*, 1998). The interaction of PSD-95 with these ion channels resulted in the redistribution of both proteins into plaque-like receptor clusters. This is very similar to the clusters of nAChR organized by rapsyn in heterologous cells (Phillips *et al.*, 1991). Unlike rapsyn however, PSD-95 molecules do not form clusters when expressed by themselves, and thus the formation of co-clusters is dependent upon the interaction between the ion channel C-terminal tail and the PDZ domain of PSD-95 (Kim *et al.*, 1996). The AMPA receptor-interacting proteins, Narp (neuronal activity-regulated pentraxin) and PICK1 (protein interacting with C kinase) both display the same clustering property when co-expressed with AMPA receptors in heterologous cells (O'Brien *et al.*, 1999; Xia *et al.*, 1999). The precise mechanism of ion channel clustering by PSD-95 has not been fully resolved. Initially, it was thought that the clustering mechanism was mediated by a simple cross-linking model in which the PDZ1 and PDZ2 domains of PSD-95 allowed the bivalent linkage of ion channels. This was supported by the fact that PSD-95 molecules did not form homomeric clusters when expressed in the absence of their associated ion channel. However, Hsueh *et al.* (1997) demonstrated that a truncated PSD-95 molecule comprising the N-terminal region and a

single PDZ domain was still able to cluster NMDA receptors, suggesting an alternative mechanism. Subsequently it has been shown that clustering of ion channels in heterologous cells is dependent upon two N-terminal cysteine residues located at amino acid positions 3 and 5 of PSD-95 (Hsueh *et al.*, 1997; Hsueh and Sheng, 1999).

Although channel clustering activity is a useful property for a protein that organizes microdomains of ion channels on the cell surface, it does not prove a clustering function for PSD-95. Operational proof of such a function requires that the ion channel becomes redistributed when its interaction with PSD-95 is abolished. Indeed, the unaltered localization of NMDA receptors to post-synaptic sites in PSD-95-knockout mutant mice suggested that this was not in fact the function of PSD-95 (Migaud *et al.*, 1998). However, the other MAGUKs may be able to compensate for the loss of PSD-95 in these mice. Perhaps the best evidence for a role of MAGUK proteins in the clustering of ion channels was provided by the *in vitro* and *in vivo* characterisations of *dlg* knockouts in *Drosophila*. Fortunately, *dlg* is the only PSD-95-like protein represented in *Drosophila* allowing genetic analysis of *dlg* function with respect to K⁺ channel localisation. In *Dlg* null mutants, the normal synaptic localization of K⁺ channels was severely disrupted despite expression of normal levels of channel protein (Budnik *et al.*, 1996; Tejedor *et al.*, 1997). This demonstrated the clear requirement for *dlg* in synaptic clustering of its partner ion channel. Further, deletion mutants were able to distinguish between ion channel clustering (that is aggregation of ion channels) and subcellular targeting of ion channels to synaptic sites. The deletion of the GK and the PDZ3 domain of *dlg* did not effect the aggregation of K⁺ channels into protein rich domains, but resulted in the clustering of these channels at non-synaptic sites. These results suggest that indeed the PSD-95 family of proteins may be responsible for both the aggregation of receptors in the PSDs of mammalian brains and the targeting of these receptors to synaptic sites. It follows therefore that PSD-95 proteins themselves must be targeted to the PSD and may interact with additional structural proteins.

With respect to the targeting of PSD-95 proteins to synaptic sites a number of groups have investigated possible domains within PSD-95 which mediate its synaptic localization. GFP-tagged-PSD-95 expressed in cultured hippocampal neurons was localized to synaptic sites. This localization was shown to be dependent upon the first two PDZ domains, a consensus YXX ϕ (where Y is tyrosine X is any amino acid and ϕ is a hydrophobic amino acid) trafficking motif located in the C-terminus and palmitoylation of its two N-terminal cysteines (Arnold and Clapham, 1999; Craven *et al.*, 1999; Craven *et al.*, 2000; El-Husseini *et al.*, 2000a). Interestingly the other isoforms of PSD-95 differ in

their synaptic localization in cultured neurons and in their ability to cluster ion channels in heterologous cells. These differences appear to be due to sequence variations in their N-terminal domains. PSD-93, which displays a similar N-terminal domain to PSD-95, recruits Kv1.4 K⁺ channels into cell surface clusters in COS cells whereas SAP97 and SAP102 also cluster Kv1.4 into receptor rich domains but intracellularly (Kim and Sheng, 1996; Tiffany *et al.*, 2000; El-Husseini *et al.*, 2000a). SAP97 lacks the two N-terminal cysteines and is therefore not palmitoylated, whereas SAP102 does contain the two cysteines. The lack of cell surface clustering displayed by SAP102 is thought to be due to non-palmitoylation of these two cysteine residues (El-Husseini *et al.*, 2000b). In cultured hippocampal neurons both PSD-95 and PSD-93 are targeted to post-synaptic sites whereas SAP97 and SAP102 are distributed to the dendrites and axons.

1.12.2 Cytoskeletal attachment of NMDA receptors

The role of the PSD-95 family of proteins in clustering and targeting of NMDA receptors at post-synaptic sites, is undoubtedly complex. During development, it is not known whether PSD-95 targets NMDA receptors to synaptic sites or whether the receptors are localized to the PSD even before PSD-95 itself is targeted there. It is possible that PSD-95 merely tethers NMDA receptors to the PSD once they are targeted to the post-synaptic membrane via some other regulatory pathway. Indeed, as shown by genetic studies, PSD-95 is definitely not the only protein capable of localising NMDA receptors to the synapse. In any case, it is clear from the specific concentration of receptors at post-synaptic sites that NMDA receptors are tethered to cytoskeletal proteins directly, via the PSD-95 family of proteins, or via other interacting proteins. The importance of this cytoskeletal attachment for NMDA receptor function was demonstrated by Rosenmund and Westbrook (1993). They reported that the Ca²⁺-dependent rundown of NMDA receptor channels observed in whole-cell and inside-out patches from cultured hippocampal neurons was prevented when actin depolymerization was blocked. Furthermore, Allison *et al.* (1998) showed that depolymerization of F-actin in hippocampal cultures decreased the number of synaptic NMDA receptor clusters. An explanation for this activity-dependent decrease in the number of synaptic NMDA receptors was that the specific binding of a number of cytoskeletal proteins to the NMDA receptor is regulated by Ca²⁺. The binding of both spectrin and α -actinin 2 is antagonised by Ca²⁺, suggesting that the regulated association of NMDA receptors with the cytoskeleton may be a mechanism contributing to synaptic

plasticity (Wyszynski *et al.* 1997; Wechsler and Teichberg, 1998).

As well as the cytoskeletal proteins known to associate directly with NR1 and NR2 subunits, further yeast two-hybrid screens with defined domains of the MAGUK proteins identified a number of cytoskeletal proteins that interacted with PSD-95 and PSD-93 suggesting that NMDA receptors are potentially tethered to the PSD via a number of structural linkages (Brenman *et al.*, 1998; Neithammer *et al.*, 1998; Passafaro *et al.*, 1999). For example, CRIPT, which binds to both the PDZ3 domain of PSD-95 and to microtubules, recruits PSD-95 to microtubules in hippocampal neurons (Neithammer *et al.*, 1998; Passafaro *et al.*, 1999). In addition, the PDZ3 domain of PSD-95 is known to associate with neuroligins, which are neuronal cell surface proteins composed of an extracellular domain that resembles acetylcholinesterase, a transmembrane domain and a highly conserved intracellular sequence (Irie *et al.*, 1997). The neuroligins are known to bind pre-synaptic neuexins, and are able to form intercellular junctions. Thus, an intriguing possibility is that via these intercellular proteins, the localization of the NMDA receptor can be regulated with respect to its position opposite the pre-synaptic terminal.

Sequential yeast two-hybrid screens have identified chains of interacting proteins that ultimately link NMDA receptors and other glutamate receptors to the cytoskeleton via a number of intermediate proteins. As a result, these different chains of interacting proteins have finally converged such that an indirect association between NMDA receptors and mGluR has been demonstrated. The first protein identified to interact with the GK domain of PSD-95, named GKAP (guanylate kinase-associated protein), was subsequently shown to associate with a novel family of proteins, named SHANK (Takeuchi *et al.*, 1997; Kim *et al.*, 1997; Naisbitt *et al.*, 1997; Naisbitt *et al.*, 1999). At the other end of the chain, the mGluR-associated protein, Homer-1c, interacts with SHANK (Tu *et al.*, 1998; Tu *et al.*, 1999). Both SHANK and GKAP interact with the cytoskeletal proteins, cortactin, in the case of SHANK, and myosin-V and dynein, in the case of GKAP (Naisbitt *et al.*, 2000). Therefore, a cytoskeletal link between NMDA receptors and mGluR has been established suggesting a biochemical mechanism for functional cross-talk between these different glutamate receptors.

Recently proteomic analysis of an NMDA receptor adhesion complex immunoprecipitated from mouse brain extracts identified 16 cell-adhesion and cytoskeletal proteins that were co-associated either directly or indirectly with the NR1 subunit (Husi *et al.*, 2000). Precisely which proteins interact with which and the relative importance of each protein to the tethering of the NMDA receptor to the PSD remains to be elucidated.

Genetic approaches to study the role of possible interactions of NMDA receptors with both cytoskeletal and regulatory proteins have avoided the problems of multiple NMDA receptor-interacting proteins by generating transgenic mice expressing NMDA receptors without the large intracellular C-terminal region of any one of the three NR2 subunits (Mori *et al.*, 1998; Sprengel *et al.*, 1998; Steigerwald *et al.*, 2000). The importance of the NR2A and NR2B C-terminal domains was illustrated by the fact that mutant mice expressing either truncated NR2A or NR2B resembled the respective NR2 knockout mice (Forrest *et al.*, 1994; Li *et al.*, 1994). With respect to the localization of the NMDA receptors in these mice, there was a reduced number of synaptic NR2A-truncated-containing receptors compared to the wild-type (Steigerwald *et al.*, 2000). However, the number of somatic, *i.e.* extra-synaptic mutant receptors appeared unchanged. The NR2 C-terminus was therefore crucial for the synaptic localization of NMDA receptors presumably due to its multiple binding partners located in the PSD implying that the association of NMDA receptors with cytoskeletal proteins via the PSD-95 family is at least as important for the synaptic localization of receptors as the association of the NR1 subunit with cytoskeletal proteins.

1.12.3 Regulation of ion channel function

The identification of the PSD-95 family of MAGUK proteins raised the possibility that the regulated association of ion channels with intracellular proteins was a mechanism for controlling channel function. A number of preliminary investigations were therefore carried out to determine whether a direct association of MAGUK proteins with either NMDA receptors, kainate receptors or K⁺ channels in heterologous cells, changed the function of the targeted ion channels.

The results of a few studies showed that the association of PSD-95 with NMDA receptor NR2 subunits and with GluR6/KA2 receptors altered the electrophysiological properties of these ion channels. The binding of PSD-95 to recombinant kainate receptors altered receptor function by reducing desensitization (Garcia *et al.*, 1998), whereas the binding to $\zeta 1/\epsilon 2$ NMDA receptors decreased the sensitivity of current responses to L-glutamate (Yamada *et al.*, 1999).

1.12.4 Recruitment of neuronal signalling molecules

The role of PSD-95 in synaptic plasticity

The PSD-95 family of MAGUKs and their associated proteins are thought to function as scaffolding molecules for the recruitment of the many proteins known to be responsible for both the regulation of NMDA receptors and for the Ca^{2+} -mediated stimulation of neuronal signalling pathways. The importance of these interactions was demonstrated with mutant mice carrying C-terminally truncated NR2 subunits. These truncated subunits failed to participate in some physiological events such as LTP, indicating that NMDA receptor activation was disconnected from the signal transduction pathways responsible for NMDA receptor-mediated changes in synaptic transmission (Sprengel *et al.*, 1998; Mori *et al.*, 1998; Steigerwald *et al.*, 2000). PSD-95 knockout mice also exhibited overactive LTP such that the frequency of stimuli required to induce LTP was significantly reduced compared to wild-type mice suggesting the involvement of PSD-95 in signalling pathways responsible for the induction of LTP (Migaud *et al.*, 1998). The question of what proteins comprise the signalling pathway downstream of the PSD-95-like-associated proteins is now the focus for many molecular neuroscientists. In fact many clues to the nature of these signalling proteins have been provided by the identification of PSD-95-interacting proteins.

The participation of PSD-95 in signalling cascades

Many of the downstream proteins that were cloned as a result of both the yeast two-hybrid screens using various protein domains of PSD-95 and the biochemical analysis of NMDA receptor-associated protein complexes (Husi *et al.*, 2000), are potential regulators of signal transduction pathways. PSD-95 binds both GTPase-activating proteins (GAPs), e.g. synGAP (synaptic GTPase-activating protein) and citron, and GDP-GTP exchange proteins (GEPs) that regulate the activity of the small G proteins ras, rap and rho (Kim *et al.*, 1998; Chen *et al.*, 1998; Zhang *et al.*, 1999; Furuyashiki *et al.*, 1999). In addition, the protein S-SCAM (synaptic scaffolding molecule), which interacts directly with both the NMDA receptor and PSD-95 is known to bind the GEP, RapGEP (Hirao *et al.*, 1998; Hirao *et al.*, 2000; Ohtsuka *et al.*, 1999). The small G proteins are known to regulate the activity of Raf kinase, a serine/threonine kinase which in turn regulates the mitogen-activated protein kinase (MAPK) pathway. As described in section 1.7, this pathway has been implicated in various forms of synaptic plasticity resulting from NMDA receptor-

mediated Ca^{2+} entry (reviewed in Impey *et al.*, 1999). A link between PSD-95 and Raf kinase, the activator of the MAPK pathway, via the PSD-95-interacting protein MAGUIN (membrane-associated guanylate kinase-interacting protein) has also been demonstrated (Yao *et al.*, 1999; Yao *et al.*, 2000). Other GEP and GAP proteins are known to associate with the NMDA receptor signalling complex (Husi *et al.*, 2000), but detailed discussion of these is beyond the scope of this introduction. However, it is clear that the activators/inhibitors of small G proteins, the G proteins themselves and the G-protein-activated kinases are all assembled by the PSD-95 family of MAGUKs and additional PDZ containing proteins. Furthermore, some of these activators of small G proteins are potentially regulated by NMDA receptor activation. For example, the rasGTPase activity of synGAP is inhibited by CaMK-II phosphorylation which could result in the activation of the MAPK pathway. It is not surprising therefore, that the deletion of PSD-95 in transgenic mice, altered the NMDA receptor-mediated changes in synaptic plasticity.

The regulation of nNOS

In addition to the MAP kinase signalling pathway, the PSD-95 family of proteins are linked to the nitric oxide signalling pathway. NO is an important signalling molecule in both the peripheral nervous system and the CNS, and has been implicated in several forms of synaptic plasticity (reviewed in Schuman and Madison, 1994; Brenman and Brecht, 1997). The role of NO in the central nervous system has received great attention due to the potential neurotoxic effects of NO in the brain. It is thought that NMDA receptor activation may be responsible for the activation of nNOS via Ca^{2+} entry into the neuron, and therefore maybe the first step in both the activation of NO signalling pathways and in NO-mediated neurotoxicity. The co-localization of nNOS to post-synaptic sites (Weiss *et al.*, 1998) suggested that nNOS may be functionally linked to NMDA receptors. In fact, PSD-93 was first cloned with the yeast two-hybrid system using the single PDZ domain of nNOS which was subsequently shown to bind both PSD-95 and PSD-93 via their PDZ2 domains (Brenman *et al.*, 1996a; Brenman *et al.*, 1996b). Although the activity of nNOS is thought to be regulated by calmodulin (Garthwaite and Boulton, 1995), a functional coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 has been demonstrated by Sattler *et al.* (1999). They showed that by suppressing PSD-95 expression with the use of an anti-sense oligonucleotide, Ca^{2+} -activated NO production by NMDA receptors was selectively blocked. This implied that the association of PSD-95 with nNOS may govern important physiological and pathological aspects of neuronal functioning. Interestingly, an

additional PSD-95-associated protein, carboxy-terminal PDZ ligand of nNOS (CAPON), competes with nNOS for binding to PSD-95, and may therefore regulate the coupling of NMDA receptor stimulation to nNOS activity (Jaffrey *et al.*, 1998).

PSD-95 and protein phosphorylation

The participation of the PSD-95 family of proteins in the regulation of NMDA receptor function by phosphorylation and in the NMDA receptor-dependent signalling pathways initiated by protein kinases and phosphatases has been investigated by a number of research groups. As described in section 1.2.3, the NMDA receptor is highly regulated by protein kinases and protein phosphatases, and is phosphorylated at multiple sites both *in vitro* and *in vivo*. However, proof of the biochemical mechanisms responsible for the regulation of receptor function by these enzymes, required that these kinases and phosphatases be both anchored in the PSD and at some point be associated with NMDA receptors. The early investigations had identified an association of NMDA receptors with the protein tyrosine kinase, src, although it was not known whether this interaction was a direct one (Yu *et al.*, 1997). In addition, it was not known whether the actions of protein kinases and phosphatases on NMDA receptor activity were actually due to a direct phosphorylation of either NR1 or NR2 subunits. A few recent studies have demonstrated coupling of protein kinases and phosphatases to NMDA receptor complexes via the association with a variety of NMDA receptor-interacting proteins. Furthermore, the activity of some of these enzymes is regulated via this association. Yamada *et al.* (1999) reported that as well as reducing the sensitivity of NMDA receptors to L-glutamate, PSD-95 prevented the PKC-induced potentiation of $\zeta 1/\epsilon 2$ NMDA receptor responses. In agreement with this study, PSD-95 abolished the insulin potentiation of NR1-1a/NR2A receptor currents in *Xenopus* oocytes (Liao *et al.*, 2000). As described in section 1.4.4, the action of insulin is thought to be mediated by PKC, a known potentiator of NMDA receptor. The same group also reported an opposite effect of PSD-95 on the src-mediated potentiation of NR1-1a/NR2A receptor currents such that src potentiation required the presence of PSD-95. These results implied that PSD-95 was somehow involved in regulating phosphorylation of NMDA receptors by both PKC and src tyrosine kinase. An explanation for these effects of PSD-95 was offered by Tezuka *et al.* (1999). They demonstrated an association of Fyn tyrosine kinase with NMDA receptors via a direct interaction with the PDZ3 domain of PSD-95. Furthermore, the association of PSD-95 with Fyn promoted tyrosine phosphorylation of NR2A subunits in HEK 293 cells. This suggested that PSD-95

was essential for the regulation of NMDA receptor activity by Fyn and other Src-family PTKs, serving as a molecular scaffold for anchoring these PTKs to NR2A. In agreement with this role of PSD-95 as a scaffolding molecule, Kawachi *et al.* (1999) demonstrated an additional interaction of PSD-95 with the protein tyrosine phosphatase, ζ /RPTP β via the PDZ2 domain of PSD-95, although a link between this association and NMDA receptor dephosphorylation was not shown. A recent study reported the targeting of PKA to NMDA receptors via the binding of PSD-95 to A kinase anchoring protein 79 (AKAP79; Colledge *et al.*, 2000). Recruitment of other phosphatase enzymes to NMDA receptor complexes also occurs via a non-PSD-95-like-associated protein, yotiao. This, large coiled-coil protein interacts with the alternatively spliced C1 cassette of the NR1 subunit and functions to recruit both PKA and PPI to the NMDA receptor (Westphal *et al.*, 1999; Feliciello *et al.*, 1999).

The phosphorylation of ion channel receptors mediated by receptor-associated proteins may function not only to regulate the activity of the ion channel. Phosphorylation of both K⁺ channels and the AMPA receptor subunit GluR2 regulates their association with PSD-95 and PICK1 respectively, and may have important consequences on the localization and activity of both ion channels (Cohen *et al.*, 1996; Chung *et al.*, 2000). This highlights yet another possible mechanism by which receptor-associated proteins could couple neuronal signalling pathways to local changes in synaptic function.

1.13 THE AIMS OF THIS THESIS

At the start of this research project the field of NMDA receptor-associated proteins was in its infancy. A few investigations into the functions of PSD-95 had identified its role as a clustering protein and a scaffolding molecule. Furthermore, the domains of PSD-95 involved in the clustering of NMDA receptors and in the recruitment of cytoskeletal and signalling proteins had been analysed in detail. However, few studies had investigated the possible effect of PSD-95 on the biochemical and radioligand binding properties of cloned NMDA receptors. The effects of other receptor-associated proteins, namely rapsyn, gephyrin and the *Drosophila* PSD-95 homologue, *dlg*, on their corresponding binding partners suggested that the co-expression of PSD-95 with cloned NMDA receptors may have important consequences on NMDA receptor function. Therefore, a detailed analysis of the effect of PSD-95 on cloned NMDA receptors may give important clues to the function of PSD-95. Furthermore, many of the studies into the

pharmacological properties of NMDA receptors have been performed on cloned receptors expressed in non-neuronal cell lines. These host systems do not express the accessory proteins described above. The properties found may not therefore be representative of native synaptic receptors since the co-association with, for example, PSD-95 may restrict conformational changes within the receptor, or the association of PSD-95 with other proteins such as kinases, may have important influences on receptor pharmacology. Therefore, the aim of the project was to analyse the effect of the co-expression of PSD-95 with NMDA receptors in HEK 293 cells and to compare the properties of the resulting receptor complexes with those of native rat forebrain NMDA receptors. Without knowing what effect PSD-95 may have on the properties of cloned NMDA receptors, the initial focus of the project was to characterise the expression of PSD-95 in HEK 293 cells, to analyse the level NMDA receptor subunit expression in the presence and absence of PSD-95, and to investigate the effect of PSD-95 on the radioligand binding properties of heteromeric NR1/NR2 receptors.

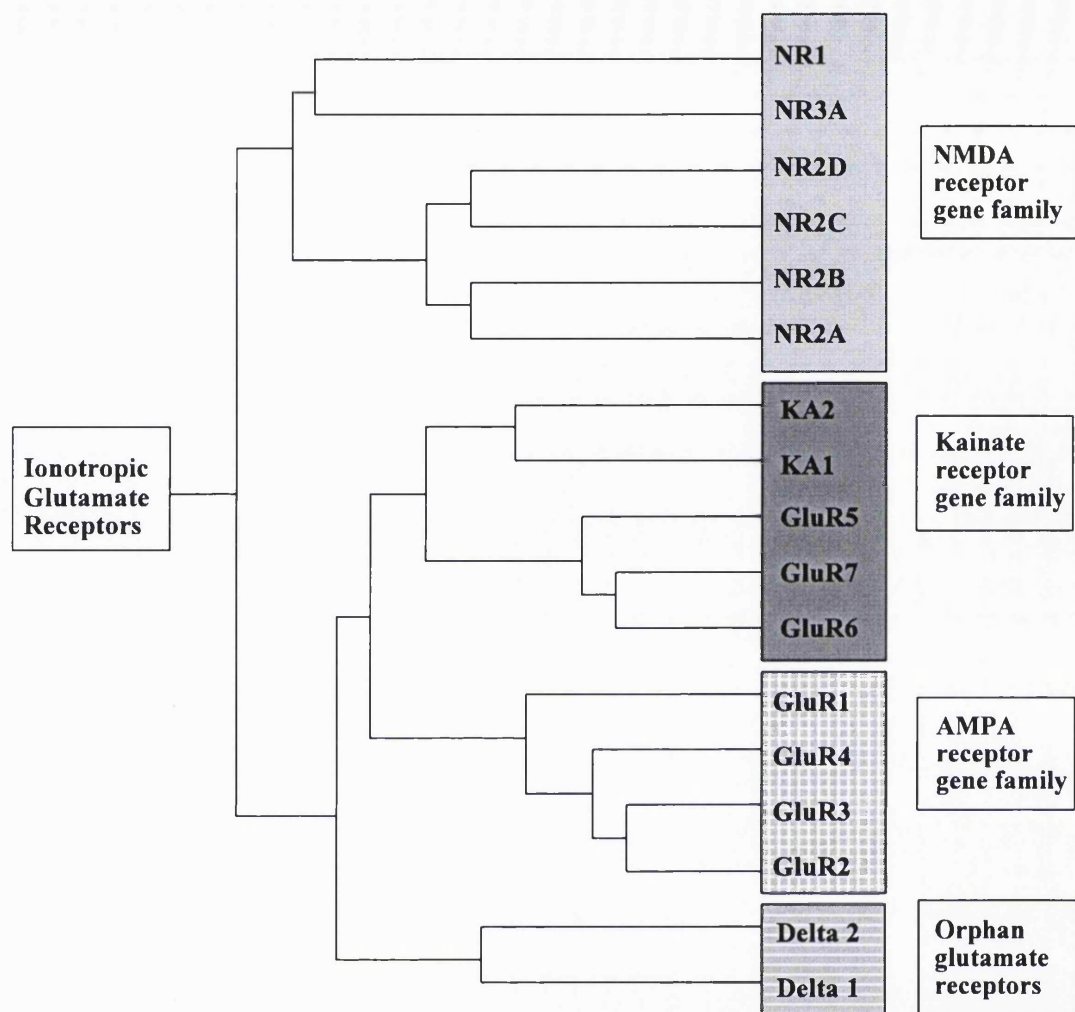


Figure 1.1 Schematic tree diagram showing the ionotropic glutamate receptor family

The diagram shows clusters of relationships among ionotropic glutamate receptor subunits created by progressive, pairwise alignment of amino acid sequences (Sucher *et al.*, 1996).

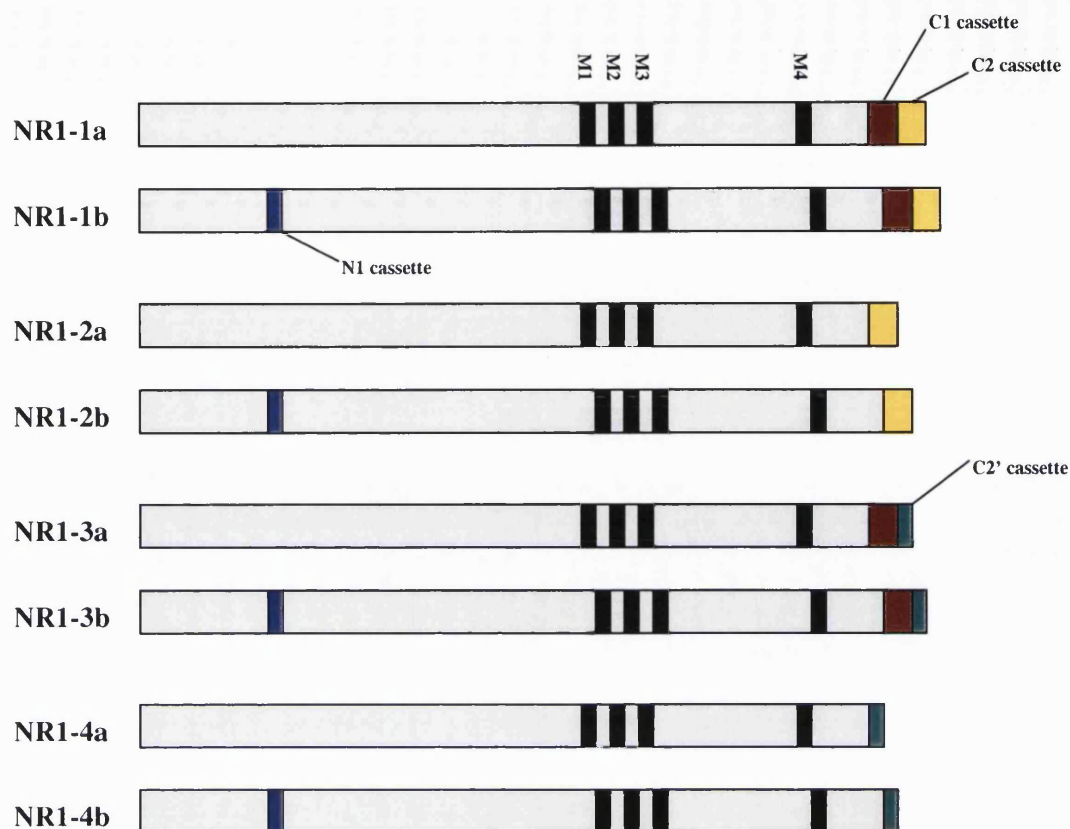


Figure 1.2 Schematic representation of the alternatively spliced NR1 subunits

The NR1 gene has a total of 22 exons, three of which undergo alternative splicing to yield theoretically eight NR1 splice variants. Exon 5 encodes a splice cassette of 21 amino acids termed N1, that is inserted at amino acid residue 190 of all the NR1b splice variants. Exons 21 and 22 encode two independent, consecutive cassettes of 37 amino acids (C1) and 38 amino acids (C2) respectively. Splicing out of the C2 exon removes the first stop codon resulting in a new open reading frame that encodes an unrelated sequence of 22 amino acids (C2') before a second stop codon is reached. The eight possible variants generated by alternative splicing of the NR1 gene are labelled according to the nomenclature of Nakanishi *et al.* (1992). The schematic structure also shows the positions of the four putative membrane domains (M1-M4) of the NR1 subunit.

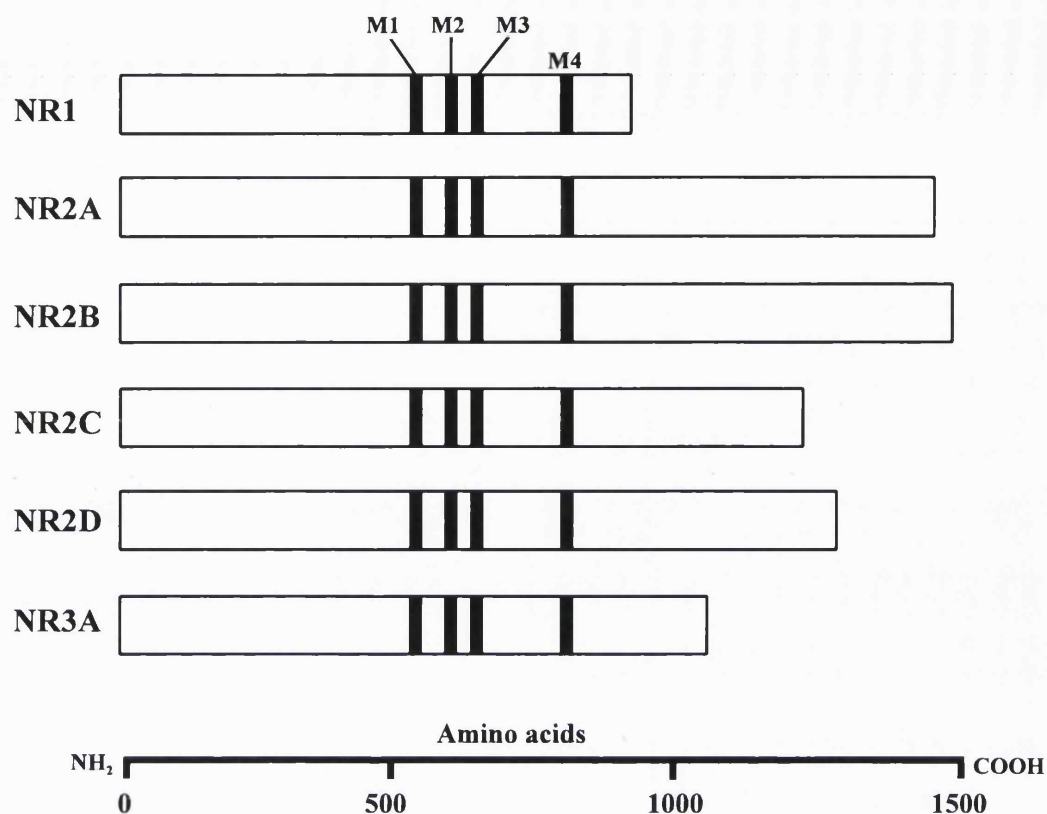


Figure 1.3 Schematic representation of the NMDA receptor subunits

All six NMDA receptor subunits display four putative hydrophobic membrane domains (M1 - M4), but differ considerably in the length of their C-termini. The Figure shows the positions of the membrane domains and highlights the variation in the lengths of the C-terminal regions among the NMDA receptor subunits.

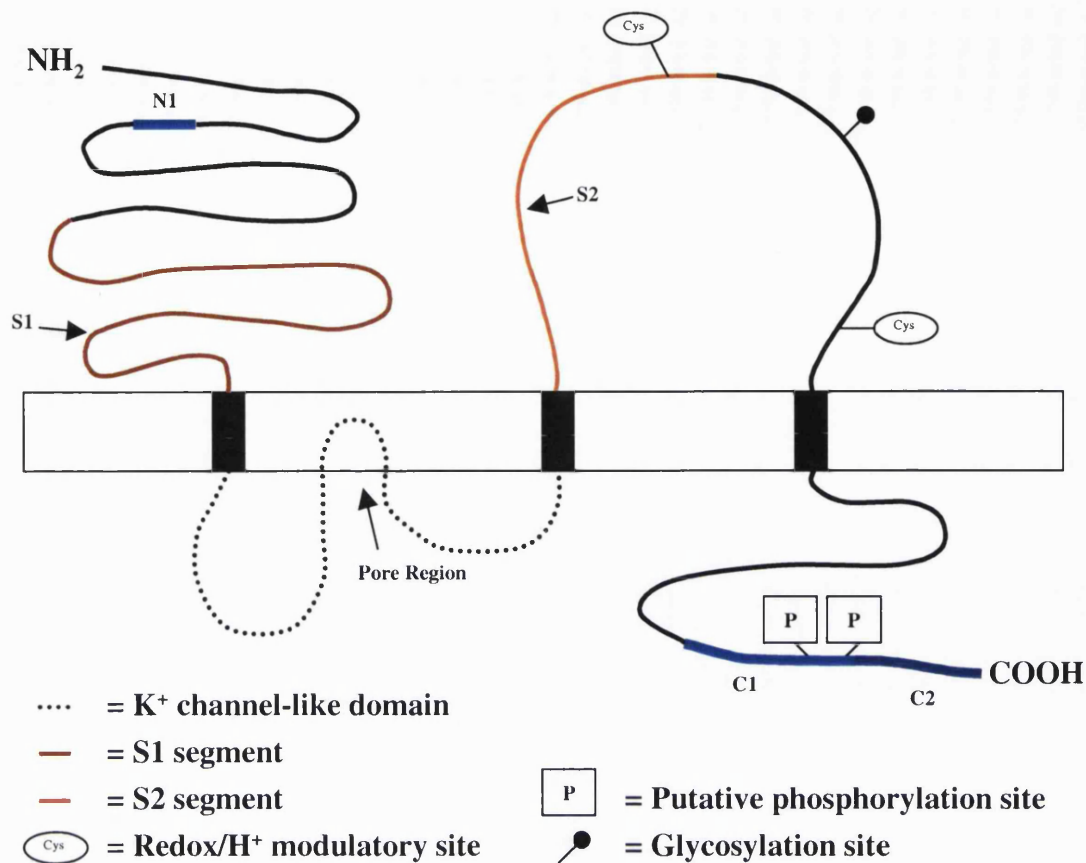


Figure 1.4 Membrane topology of the NMDA NR1 subunit

Features of the predicted NR1-protein structure include: **1.** A large extracellular N-terminus domain comprising approximately half of the protein mass; **2.** Three transmembrane spanning domains; **3.** A fourth hydrophobic segment that is thought to form a hairpin loop within the membrane. This re-entrant loop also constitutes the pore forming region of the NMDA receptor ion channel; **4.** Two extracellular segments (S1 and S2) that are predicted to form the binding domains for L-glutamate and glycine; and **5.** An intracellular carboxy-terminus domain containing three serine residues that are phosphorylated by PKA and PKC. The approximate positions of the N1, C1 and C2 exons are shown in the schematic. In addition the amino acids thought to form the redox/H⁺ modulatory site and glycosylation sites are shown.

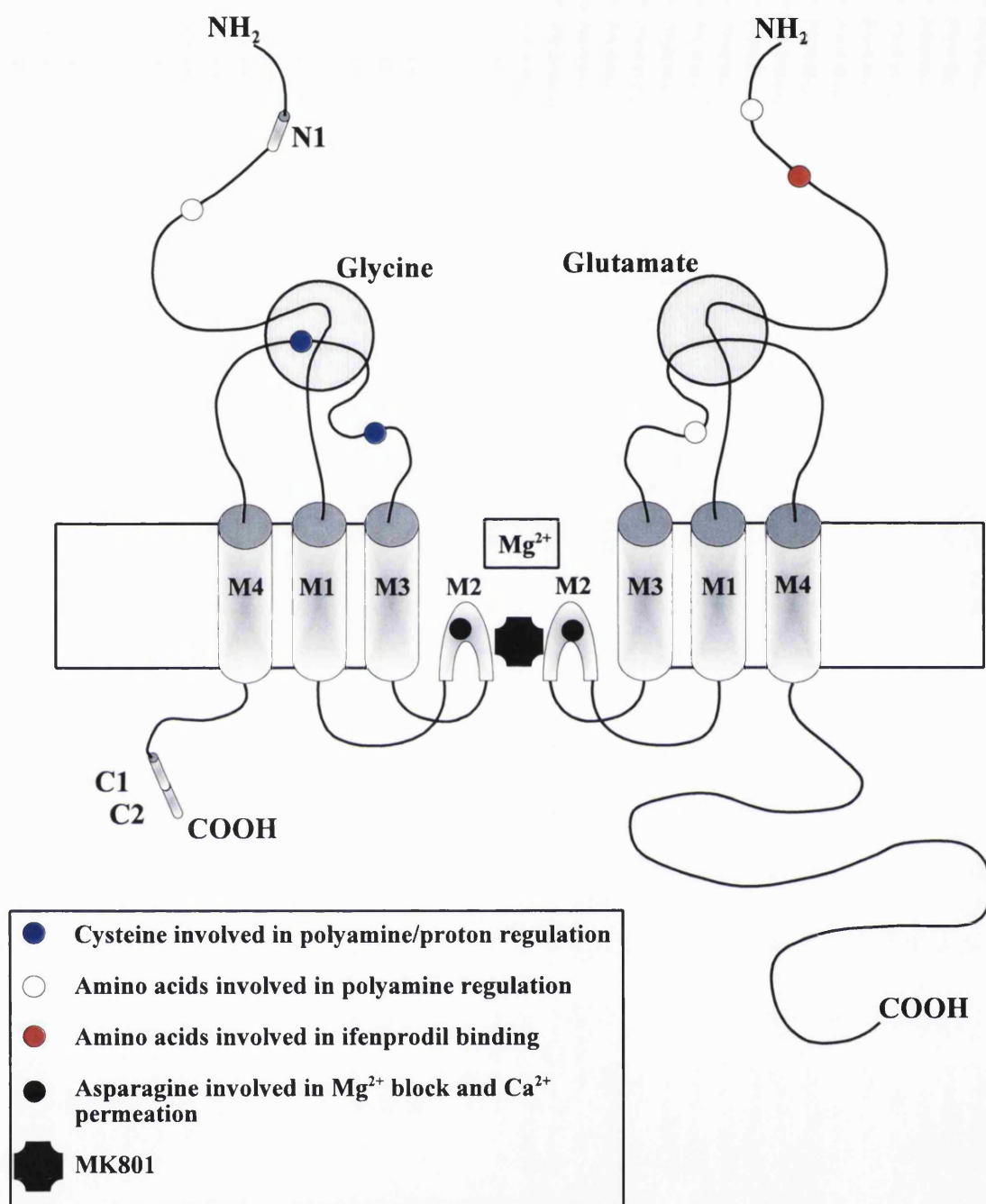


Figure 1.5 Schematic representation of the NR1/NR2B receptor showing ligand binding domains and sites of allosteric modulation

The schematic shows the location of the L-glutamate, glycine, MK801 and ifenprodil recognition sites. In addition, the location of amino acids thought to be involved in the allosteric modulation of NMDA receptors by polyamines, protons and Mg^{2+} are shown.

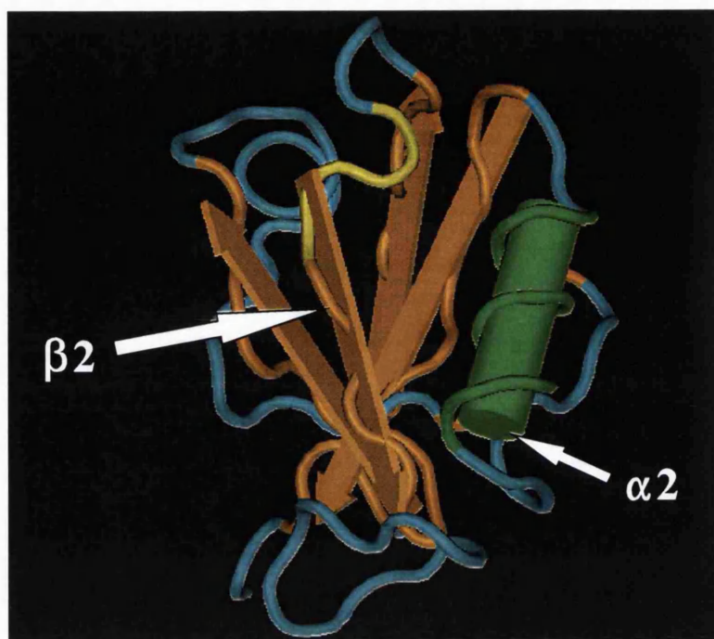


Figure 1.6 Ribbon diagram for the three-dimensional structure of the PDZ2 domain of PSD-95

The solution structure for the PDZ2 domain of PSD-95 encompassing the amino acid residues 155-249 was elucidated to high resolution by NMR spectroscopy (Tochio *et al.*, 2000). The protein contains two α helices and six β strands. The tSXV peptide binding groove is formed by the β 2 strand (labelled), the α 2 helix (highlighted in green) and the conserved glycine-leucine-glycine-phenylalanine (GLGF) sequence (highlighted in yellow). The three-dimensional structure, *i.d.* code IQLC, was taken from the NCBI protein structure database (PDB) and the figure was generated using Cn3D viewing software.

NR1		NR2	
α -Actinin 2		α -Actinin 2	(NR2B)
Spectrin		Spectrin	(NR2A/2B)
Tubulin		Tubulin	(NR2B)
NF-L	(C1 cassette dependent)	CaM-KII	(NR2A/2B)
Yotiao	(C1 cassette dependent)	PSD-95/SAP90	(NR2A/2B)
Calmodulin		PSD-93/Chapsyn 110	(NR2A/2B)
		SAP102	(NR2A/2B)
		SAP97	(NR2A/2B)
		Phospholipase C	(NR2A/2B)
		S-Scam	(NR2A/2C)

Figure 1.7 First generation NMDA receptor-interacting proteins

The table summarises proteins known to interact directly with NMDA receptors either via the NR1 or the NR2 subunit. NR1 splice variant specific and NR2 subunit subtype selective interactions are indicated on the right-hand side of the appropriate column.

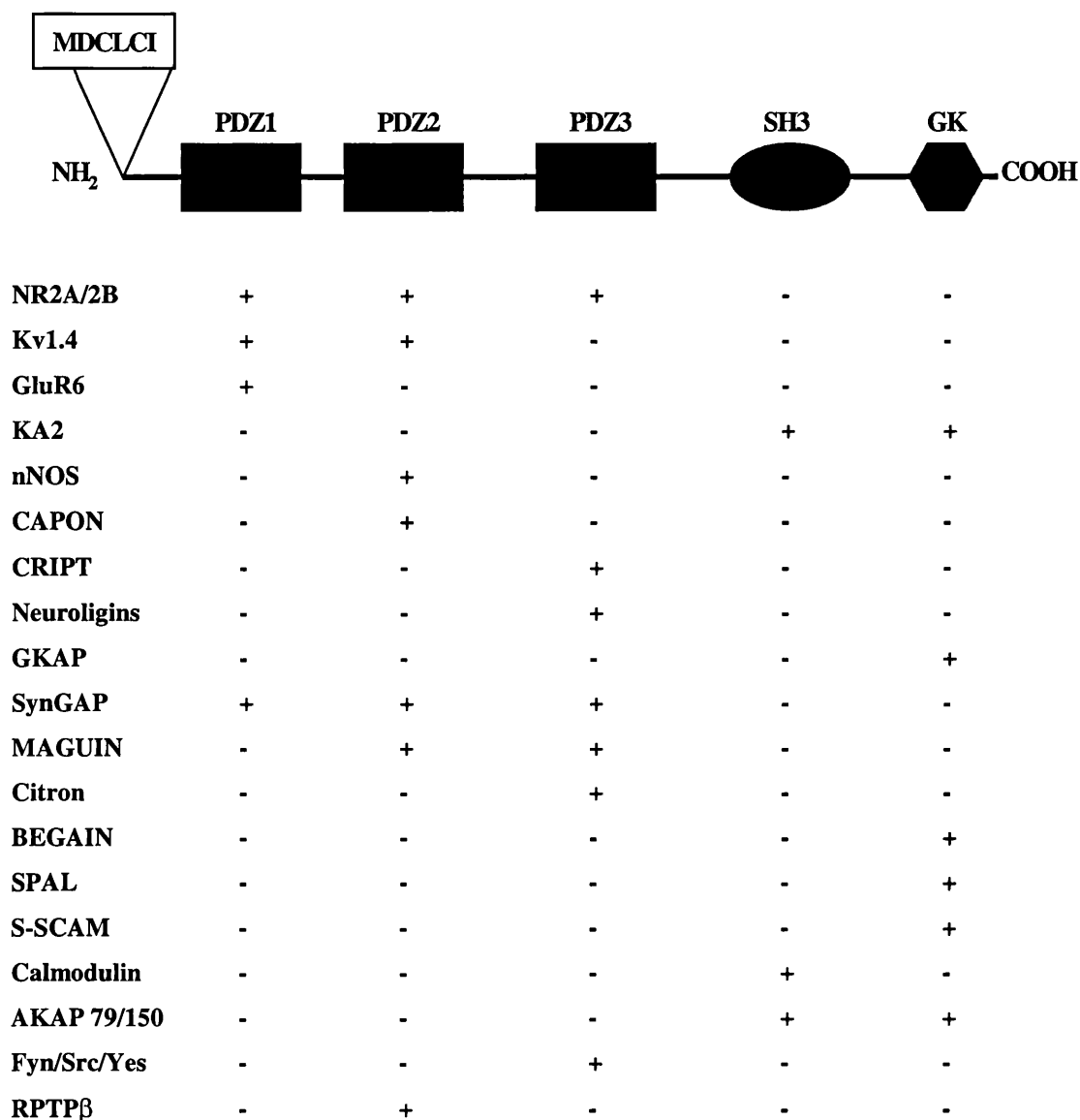


Figure 1.8 Schematic representation of PSD-95 and summary of the second generation NMDA receptor-interacting proteins

The primary structure of PSD-95 displays sequence domains typical to the PSD-95 family of membrane associated guanylate kinases. These features include: **1.** Three 90 amino acid repeats termed PDZ domains; **2.** A src homology 3 (SH3) domain; **3.** A guanylate kinase-like (GK) domain; and **4.** Two N-terminal cysteine residues located at amino acid positions 3 and 5. The location of these domains are shown in this schematic. The downstream binding partners for PSD-95 are listed below the appropriate domain of interaction. Abbreviations are shown in full in the main text or in the list of abbreviations.

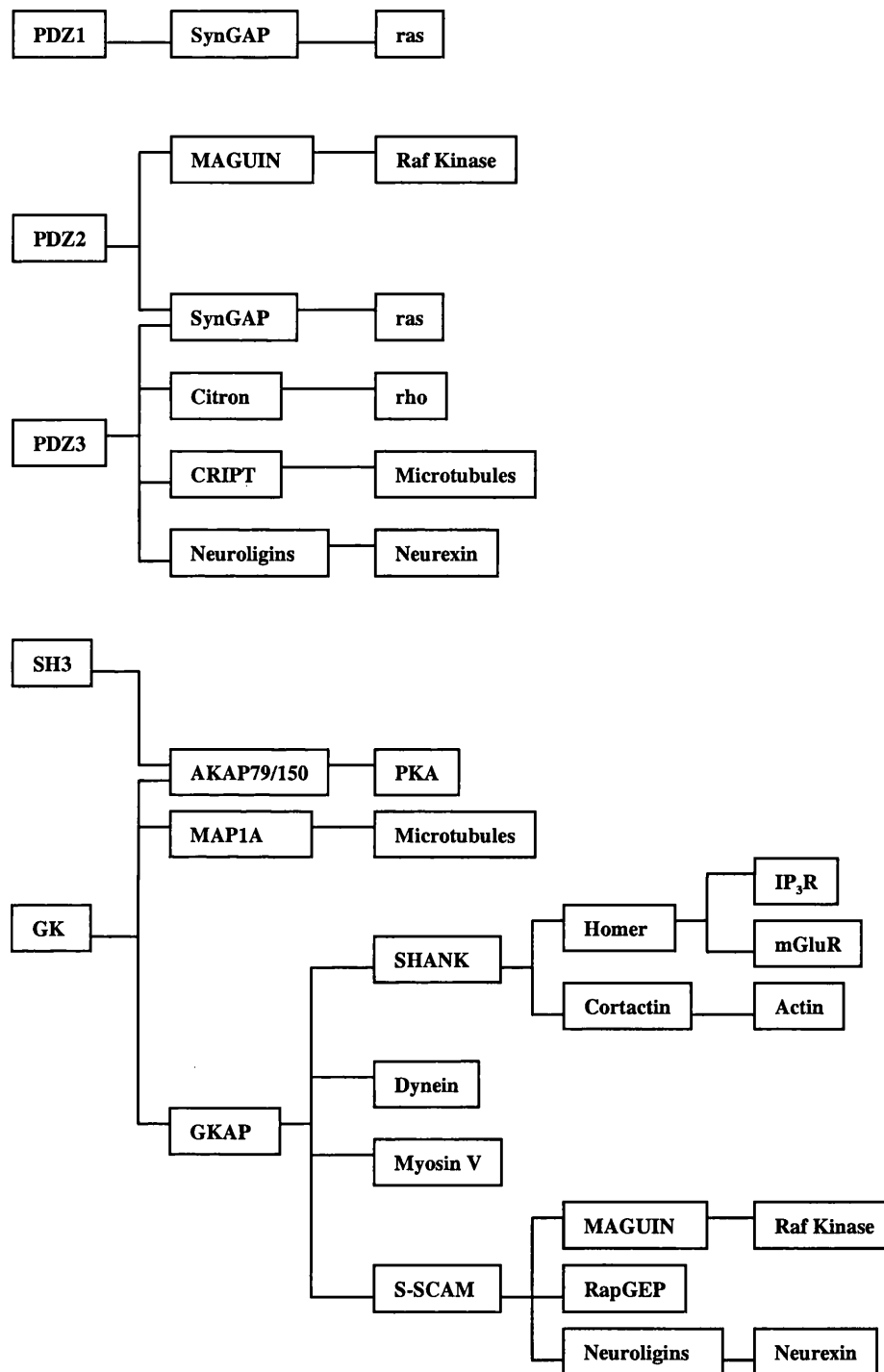


Figure 1.9 The third generation NMDA receptor-interacting proteins

Using the yeast two-hybrid system, a third generation of NMDA receptor-interacting proteins has been identified that associate with the binding partners of PSD-95, and with additional downstream proteins. The column on the left refers to the individual domains of PSD-95. For simplicity, PSD-95-associated proteins with no known additional binding partners are not shown. The reader is referred to Figure 2.7 for the full list of PSD-95-associated proteins.

CHAPTER 2

Materials and Methods

2.1 MATERIALS

Bacterial *E.coli* strain **TOP 10**: *F⁻ mcr AΔ (mrr-hsd RMS-mcr BC) ϕ80 lac ZA M15Δ lac X74 deoRrec A1, araD139Δ (ara-leu) 7697 gal u gal k rpsL end A1 nup G*, was purchased as a bacterial stab from Promega Ltd. (Southampton, U.K.). The bacterial culture media components, bactotryptone, bacto-ISP Medium 1 and bacto-agar, were from Difco Labs. (East Mosely, U.K.). Restriction enzymes, T4 DNA ligase, enzyme buffers, ampicillin, and the plasmid, pSV-β-galactosidase, were obtained from Boehringer Mannheim GmbH. (Lewes, East Sussex, U. K.). DNA molecular weight markers (GeneRuler™ 1 kb DNA ladder) were from Fermentas (Sunderland, U. K.). Ultrapure agarose (electrophoresis grade), pre-stained protein standards, oligonucleotide primers, Penicillin/Streptomycin, and sterile fetal calf serum (FCS) were obtained from GIBCOBRL Life Technologies Ltd. (Paisley, U. K.). QIAGEN maxi- and mini-plasmid purification kits, and the QIAEX™ DNA cleanup resin were obtained from QIAGEN Ltd. (Crawley, West Sussex, U.K.). The QuikChange™ site-directed mutagenesis kit was from Stratagene (Gebouw, Netherlands). The Sculptor™ *in vitro* mutagenesis kit, ECL^{plus} detection kit, Hyperfilm™ ECL, [³H]MDL 105,519 (85 Ci/mmol), horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin (Ig) antibodies, and HRP linked anti-mouse Ig antibodies were from Amersham Pharmacia (Aylesbury, Bucks., U. K.). Bio-Spin Chromatography columns were from Bio-Rad (Herts. U.K.). Dulbeccos Modified Eagle Medium/Nutrient F-12 Ham (DMEM/F-12 1:1 mixture), trypsin-EDTA, Hank's balanced salt solution, anti-c-Myc 9E10 mouse monoclonal antibodies, Protein A Sepharose, were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). The Cytotox96™ kit was from Promega Ltd. (Southampton, U.K.). Acrylamide and N'N'-methylenebisacrylamide were from BDH Laboratory Supplies (Poole, Dorset, U.K.). [³H]MK801 (21-24 Ci/mmol) and [³H]CGP 39653 (40 Ci/mmol) were from NEN (Hounslow, U.K.). MK801, ketamine hydrochloride and TCP were from RBI Ltd. (St. Albans, Herts., U.K.). AP5 and 7-CK were from Tocris Neuramin (Bristol, U.K.). Sodium orthovanadate was from Calbiochem (Nottingham, U.K.). Anti-phosphoserine, anti-phosphotyrosine cocktail (PY-20, PY-7E1, PY-1B2) and anti-phosphothreonine antibodies, and phosphotyrosine inhibitor were from Zymed Laboratories Inc. (Cambridge, U.K.). All other reagents were obtained from commercial sources.

The PSD-95 peptide (cys-480-498), CSKRRVERRE, used for antibody and affinity column production was from Immune Systems (Bristol, U.K.). HEK 293 cells were

a gift from Prof. T. G. Smart (School of Pharmacy, London, U.K.). The pGW1PSD-95_{c-Myc} and pGW1PSD-95 plasmid clones were a gift from Dr. M. Sheng (Massachusetts General Hospital, Boston, U.S.A.). NMDA receptor cDNA clones were generous gifts from Professors S. Nakanishi (Kyoto, Japan) and M. Mishina (Nigata, Japan). The pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, CA, U.S.A.).

2.2 METHODS

2.2.1 PREPARATION OF BACTERIAL GLYCEROL STOCKS

A sample of *E.coli* bacteria was cultured overnight in 10 ml Luria-Bertani (LB) media (5 g/l bactotryptone, 2.5 g/l bacto yeast extract, 5 g/l NaCl, autoclaved and supplemented with ampicillin (50 µg/ml) as necessary). For long term storage, 0.5 ml of culture was added to 0.5 ml LB media supplemented with 50 % (v/v) glycerol, and aliquoted into sterile cryogenic vials to be stored at -20°C or -80°C.

2.2.2 PREPARATION OF COMPETENT *E.COLI*

Under sterile conditions, 10 ml media A (LB media supplemented with 0.2 % (w/v) glucose and 10 mM MgSO₄, and filtered through a 0.2 µm filter) was inoculated with a sample of *E.coli* glycerol stock using a sterile loop and incubated at 37°C for 18 h with continuous shaking at 200 rpm. This overnight stock (0.5 ml) was transferred into 50 ml media A and cultured at 37°C in a shaker at 200 rpm. At mid-logarithmic phase, *i.e.* at optical density (OD)_{λ=600nm} = 0.6 - 0.8, the bacterial culture was transferred to a pre-cooled sterile falcon tube and retained on ice for 10 min. The cell suspension was pelleted by centrifugation at 3,800 x g at 4°C for 10 min and the supernatant was removed by aspiration. The pellet was resuspended in 0.5 ml of ice-cold media A by gentle pipetting and mixed with 2.5 ml media B (LB media supplemented with 36% (v/v) glycerol, 12 % (w/v) polyethyleneglycol MW8000, 12 mM MgSO₄ and filtered through a 0.2 µm syringe filter). The competent *E.coli* cells were divided into 0.1 ml aliquots and stored at -80°C for up to six months.

2.2.3 TRANSFORMATION OF COMPETENT *E.COLI*

Competent *E.coli*. were routinely transformed with either purified plasmid DNA (see section 2.2.5) or with DNA ligation reactions (see section 2.2.12). The successful transformation of recombinant plasmid DNA was selected for by the transference of antibiotic resistance to the transformed bacterial clones. An aliquot (0.1 ml) of competent *E.coli* cells was thawed on ice and either 100 ng (5 µl of a 20 ng/µl solution) of appropriate plasmid DNA or 10 µl ligation reaction (containing 50 - 75 ng DNA) was added under sterile conditions. The cell suspension was gently mixed by flicking, then left on ice for 20 min. Heat shocking was performed by placing the solution in a water bath at 42°C for 60 sec and immediately returning it to ice for a further 2 min. The cells were diluted 1:10 in LB media and incubated for 1 h at 37°C. Samples (0.1 ml) of transformed cells were plated onto LB agar plates (LB media supplemented with 1.5 % (w/v) agar and 50 µg/ml ampicillin) which were inverted and incubated for 15 - 20 h at 37°C.

2.2.4 SMALL SCALE PLASMID DNA PREPARATION

Small scale bacterial culture

A single colony of transformed *E.coli* cells was selected from the LB agar plate using a sterile loop and transferred to 2 - 5 ml LB media containing 50 µg/ml ampicillin (as appropriate). The cells were incubated for 12 - 16 h at 37°C in a shaker at 200 rpm.

Small scale plasmid purification

Plasmid purification was carried out using the QIAprep Spin Miniprep Kit protocol as described by the manufacturer (Qiagen Ltd, U.K.). A total of 3 ml of overnight bacterial cell culture was centrifuged at 12,000 x g for 5 min at room temperature (RT). The pellet was resuspended in 250 µl P1 buffer by gentle pipetting. Bacterial cell lysis was carried out with the addition of 250 µl P2 Buffer. The solution was mixed by gentle inversion 4 - 6 times and incubated until the suspension became clear, but not for more than 5 min. The solution was mixed with 350 µl N3 Buffer by gentle inversion 4 - 6 times and centrifuged at 12,000 x g for 10 min. The supernatant was collected and applied to a QIAprep spin column in a 2 ml collection tube and centrifuged at 12,000 x g for 30 - 60 sec. The flow-through was discarded and the column washed by the addition of 0.75 ml PE buffer followed by centrifugation for 30 - 60 sec. The flow-through was

discarded and the column centrifuged for an additional 1 min to remove residual wash buffer. The column was loaded with 50 µl water and incubated for 1 min, followed by centrifugation for a further 1 min into a sterile collection tube. The concentration of DNA was determined by spectrophotometric quantification as described in section 2.2.6.

2.2.5 LARGE SCALE PLASMID DNA PREPARATION

Small scale bacterial culture

Small scale bacterial cultures were prepared as described in section 2.2.4 except that incubations were carried out for 8 h at 37°C in a shaker at 200 rpm.

Large scale bacterial culture

A large scale overnight culture was prepared by adding 0.5 ml of small scale overnight culture to 500 ml LB media containing 50 µg/ml ampicillin in a sterile conical flask, loosely sealed with sterile gauze swab. The cells were cultured for 12 - 16 h at 37°C in a shaker at 200 rpm to a density of approximately 1×10^9 cells/ml, *i.e.* $OD_{\lambda=600nm} = 1$.

Maxi-scale plasmid DNA preparation

Plasmid purification was carried out using the Qiagen™ plasmid maxi-kit. The overnight culture was transferred into two JA14 centrifuge tubes and centrifuged at $3,800 \times g$ for 10 min at 4°C. The supernatant was removed by decanting and both cell pellets were completely resuspended in 10 ml of ice-cold P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 100 µg/ml RNase). Bacterial cell lysis was carried out by the addition of 10 ml P2 buffer (200 mM NaOH and 1% (w/v) sodium dodecyl sulphate [SDS]). The solution was mixed gently by inversion and incubated at RT for 5 min. The lysate was neutralised with 10 ml ice-cold P3 buffer (3.0 M potassium acetate, pH 5.5), mixed gently by inversion, and incubated on ice for 20 min. Precipitated cellular debris was pelleted by centrifugation at $27,000 \times g$ for 30 min at 4°C and the resulting supernatant was loaded onto a Qiagen™ 500 column, pre-equilibrated with 10 ml QBT buffer (50 mM 3-N-[Morpholino]propane-sulphonic acid (MOPS), pH 7.0, 750 mM NaCl, 15% (v/v) isopropanol, 0.15 % (v/v) Triton X-100). The supernatant was allowed to flow completely through the column by gravity and the resin was washed with 60 ml QC buffer (50 mM MOPS, pH 7.0, 1 M NaCl, 15% (v/v) isopropanol). Plasmid DNA was eluted from the column into a sterile JA20 centrifuge tube with 15 ml QF buffer (50 mM Tris-HCl, pH 8.5,

1.25 M NaCl, 15% (v/v) isopropanol) and precipitated with 10.5 ml isopropanol at RT. The suspension was centrifuged at 27,000 x g for 30 min at 4°C, the supernatant was removed by aspiration, and the DNA pellet rinsed gently in 2 ml of ice-cold 70 % (v/v) ethanol. The ethanol was removed by aspiration and the pellet left to air dry for 10 min. The plasmid DNA was dissolved in 300 µl TE buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA) at 4°C for 12 - 18 h. The concentration of DNA was determined by spectrophotometric quantification and a subsequent addition of TE buffer was made to dilute the plasmid to a concentration of 1 µg/µl.

2.2.6 QUANTIFICATION OF DNA CONCENTRATION AND PURITY

A 10 µl sample of purified DNA was diluted to 1 ml with TE buffer and the OD at $\lambda=260$ nm and $\lambda=280$ nm determined. An OD _{$\lambda=260\text{nm}$} = 1 corresponds to a concentration of 50 µg/ml for double stranded DNA (dsDNA), 40 µg/ml for single stranded DNA (ssDNA), and 20 µg/ml for oligonucleotides. The ratio between readings at $\lambda=260$ nm and $\lambda=280$ nm determines the purity of nucleic acid. A pure solution of DNA has a ratio of 1.8. A range of 1.8 - 2.0 was acceptable for the use of purified plasmid DNA in HEK 293 cell transfections.

2.2.7 RESTRICTION ENZYME DIGESTION

Restriction enzyme digests were performed typically on 1 - 2 µg DNA. For the digestion of larger quantities of DNA, the following reaction volumes were scaled up appropriately. Approximately 1 µg DNA was mixed with sterile water up to a volume of 17 µl. The 10 x restriction enzyme buffer (2 µl), appropriate for the particular restriction enzyme to be used, was added. The digestion reaction was initiated by the addition of 5 - 10 units (1 - 2 µl) of restriction enzyme, and then mixed by flicking, briefly centrifuged, and incubated at 37°C for 1 h.

2.2.8 FLAT-BED AGAROSE GEL ELECTROPHORESIS

Flat-bed agarose gel electrophoresis was performed using a Flowgen MH 1070 minigel apparatus. All analyses were carried out using 0.9 % (w/v) agarose gels unless otherwise stated. The following stock solutions were prepared: 10 x TBE (0.9 M Tris-HCl,

pH 8.0, 0.9 M boric acid and 40 mM EDTA, autoclaved); 6 x gel loading buffer (60 mM EDTA, pH 8.0, 0.25 % (w/v) bromophenol blue and 30 % (v/v) glycerol); 0.9 % (w/v) agarose (1 x TBE and 0.9 % (w/v) agarose) stored at 70°C after autoclaving. The minigel was prepared by supplementing 40 ml 0.9 % agarose, cooled to 40 - 50 °C, with 5 µl ethidium bromide (10 mg/ml). This gel was poured into the assembled gel apparatus and left to polymerize for 1 h at RT. The metal moulding gates and loading comb were removed and the gel was bathed in 1 x TBE to cover the gel by approximately 0.25 cm. DNA samples (approximately 250 ng) to be analysed were mixed with 2 µl 6 x gel-loading buffer and TE buffer to a final volume of 12 µl. These DNA samples and 12 µl of appropriate molecular weight standards were loaded into the gel and electrophoresis was carried out at 50 V constant voltage for 1 h. DNA bands were viewed under UV light using a Flowgen transilluminator at $\lambda = 312$ nm. Gel photography was carried out using a Kodak DC120 200 M Digital Camera.

2.2.9 GEL EXTRACTION OF RESTRICTION ENZYME FRAGMENTS BY ELECTROELUTION

Restriction enzyme digests were analysed by flat-bed agarose gel electrophoresis as described in section 2.2.8. The gel was viewed briefly under UV illumination and the appropriate fragment was excised rapidly using a sterile scalpel. An suitable length of dialysis tubing (with molecular weight cut off 40,000 Da) was selected and pre-soaked in a beaker of water for 5 min in a boiling water bath. The beaker was removed from the water bath and retained at RT until the dialysis tubing was required. The dialysis tubing was sealed at one end with a dialysis clip and loaded with 400 µl TE buffer. The piece of excised gel was placed in the tubing, air bubbles were removed and another dialysis clip attached to seal the other end. The dialysis tubing was placed in a flat-bed gel electrophoresis tray and overlaid with 50 ml 1 x TBE. Electrophoresis was carried out for 3 h at 50 V constant voltage. Complete extraction of the DNA from the gel was confirmed by brief illumination under UV light. The TE buffer was collected from the dialysis tubing and an additional 100 µl TE buffer was used to rinse any remaining DNA from the tubing.

2.2.10 PHENOL/CHLOROFORM PURIFICATION OF RESTRICTION ENZYME DIGESTION REACTIONS

Fragments of DNA generated by restriction enzyme digestion (2.2.7) to be used in DNA ligation reactions were purified by phenol/chloroform extraction. The restriction enzyme digest was mixed with sterile water to yield a final volume of 100 µl followed by the addition of 100 µl Tris-washed phenol. The mixture was vortexed and centrifuged at 12,000 x g for 5 min at RT. The upper aqueous layer was collected and transferred to a sterile tube. The volume of this aqueous phase was measured using a Gilson pipette and an equal volume of chloroform was added and vortexed. Centrifugation was performed at 12,000 x g for 5 min at RT and the upper phase was again collected and transferred to another tube. The volume of this fraction was measured and an equal volume of chloroform added. The cycle of centrifugation and upper phase collection was finally repeated.

2.2.11 ETHANOL PRECIPITATION OF DNA

DNA from the solutions generated by either the electroelution of restriction fragments or the phenol/chloroform extraction of restriction enzyme digests was precipitated with the addition of $\frac{1}{10}$ th volume of 3 M sodium acetate and 2 volumes of 100 % ethanol. The solution was mixed by inversion and incubated at -20°C overnight. Precipitated DNA was pelleted by centrifugation at 12,000 x g at 4°C for 15 min. The supernatant was discarded and the pellet was washed in 200 µl 70 % (v/v) ethanol. Centrifugation at 12,000 x g was carried out for a further 15 min at 4°C and the supernatant was again discarded. The pellet was allowed to air dry and then resuspended in an appropriate volume of water or TE buffer. DNA restriction fragments precipitated from electroeluted samples were further purified to remove any agarose remaining in the precipitated DNA solution. The agarose was removed by incubating the solution at 35°C for 5 min and immediately centrifuging the sample for 1 min at 12,000 x g at RT. The supernatant was collected and transferred to a sterile tube. The concentration of DNA yielded by ethanol precipitation was determined by comparing the intensities of 1 µl DNA sample and a known quantity of DNA analysed by flat-bed agarose gel electrophoresis.

2.2.12 DNA LIGATION REACTIONS

DNA restriction fragments were prepared for ligation reactions by electroelution from flat-bed agarose gels (2.2.8). DNA plasmids were digested with the appropriate restriction enzymes and purified by phenol/chloroform extraction (2.2.10). Both samples were ethanol precipitated and resuspended in appropriate volumes of water. Reactions were performed by incubating 100 - 150 ng vector DNA with the DNA insert at insert:vector molar ratios of 1:1, 3:1 and 10:1. The solutions were mixed with 2 μ l 10 x DNA ligase buffer (660 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 10 mM dithiothreitol (DTT), and 10 mM adenosine triphosphate (ATP)) and 1 μ l DNA ligase enzyme (1 unit/ μ l) and a final volume of 20 μ l was obtained with the addition of water. Four control reactions were performed in parallel using: unrestricted vector plus insert; restricted vector plus insert minus ligase; restricted vector minus insert plus DNA ligase; no DNA. Ligation reactions were incubated at 16°C overnight. Competent *E.coli* were then transformed with the ligation reaction (10 μ l) as described in sections 2.2.3 and 2.2.13.2.

2.2.13 SELECTION OF RECOMBINANT M13 mp18 PLASMID CLONES

Bacteria transformed with bacteriophage M13mp18 vector constructs could not be selected on the basis of transference of antibiotic resistance to the transformed bacterial cell since the M13 vector does not contain an antibiotic resistance gene. Therefore recombinant M13mp18 plasmids were selected by the bacteriophage production from transformed *E.coli* host strain, TG1 (*r_kr_m*) *supE* Δ (*lac-proAB*) *hsd* Δ 5 *F'**traD36 proAB*⁺ *lacI^q lacZ* Δ M15 (provided with the Sculptor™ *in vitro* mutagenesis kit). Phage production was detected by the formation of plaques in a lawn of non-competent TG1 cells (lawn TG1 cells). DNA fragments ligated into the M13 vector were inserted into the multiple cloning site located in the coding region for the *lacZ* gene. Thus, selection of recombinant M13mp18 clones utilized the white/blue plaque selection procedure.

2.2.13.1 Revival of *E.coli* host strain TG1 from bacterial freeze-dried discs

Glucose/minimal media agar plates were prepared for short-term storage of TG1 cell colonies. Solutions of sterile 20 % (w/v) glucose, 5 x salts (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄, 43 mM NaCl, and 9 mM NH₄Cl), and 1 mg/ml thiamine were prepared.

A solution of agar (1.5 % (w/v)) supplemented with final concentrations of 0.1 mM CaCl₂ and 1 mM MgSO₄ was autoclaved and cooled to 50°C. This was followed by the further addition of glucose to a final concentration of 0.4 % (w/v), 5 x salts diluted 1:500, and thiamine to 1 µg/ml. Plates were poured and allowed to polymerize. TG1 cells were revived from the bacterial freeze-dried discs provided by the manufacturer (Amersham Pharmacia, Bucks. U.K.) by incubating a single disc with 0.5 ml 2 x YT (8g/l bactotryptone, 5 g/l bacto ISP Medium, and 2.5 g/l NaCl, autoclaved) for 1 min with agitation. The revived TG1 cells (20 µl) were plated onto glucose/minimal media plates which were inverted and incubated for 24 - 36 h at 37°C. The plates of TG1 colonies were then retained at 4°C for future use.

2.2.13.2 Transformation of competent TG1 cells with M13mp18DNA

A small scale overnight culture of non-competent TG1 cells was generated by inoculating 10 ml 2 x YT media with a colony of cells selected from the glucose/minimal media stock. The culture was incubated overnight at 37°C in a shaker at 200 rpm. Lawn TG1 cells were prepared by diluting an appropriate volume of overnight non-competent TG1 cell culture 1:100 in 2 x YT. The cells were cultured for approximately 2 h until OD_{λ=600nm} = 0.3. Competent TG1 cells were prepared as described in section 2.2.2 and were transformed as described in section 2.2.3 with 10 µl ligation reaction but were retained on ice after the heat shock step until the lawn TG1 cells had reached the required OD. Control transformation reactions were performed using 1 ng M13 mp18 vector. A mixture of 200 µl lawn TG1 cells, 40 µl sterile 100 mM isopropylthio-β-D-galactosidase (IPTG) and 40 µl 2 % (w/v) 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) in dimethylformamide was added to an aliquot of transformed TG1 cells. This solution was then mixed with 4 - 5 ml of hand-warm Top Agar (10 g/l bactotryptone, 8 g/l NaCl and 8 g/l agar, autoclaved) and poured onto a 1.5 % (w/v) LB agar plate. Plates were inverted and incubated at 37°C for 16 h.

2.2.13.3 Generation of bacteriophage stocks

Clear plaques were collected using a 100 µl pipette tip. The resulting agar plug was ejected into 5 ml 2 x YT media containing 50 µl of an overnight non-competent TG1 cell culture. The cells were cultured for 5 h at 37°C with shaking at 200 rpm. Phage particles were collected from 1.5 ml TG1 cell culture by centrifugation at 12,000 x g for

5 min at RT. The supernatant, enriched with phage particles, was removed and stored at 4°C.

2.2.13.4 Purification of M13mp18 replicative form (RF) DNA

Competent TG1 cells were transformed as described in section 2.2.13.2. Clear plaques were selected and used to inoculate non-competent TG1 cells as described in section 2.2.13.3. Alternatively, an overnight culture of TG1 cells was inoculated with 5 µl phage stock (see section 2.2.13.3). The cells were cultured for 5 h at 37°C with shaking at 200 rpm. TG1 cells were collected from a total of 3 ml cell culture by centrifugation at 12,000 x g for 5 min. The supernatant was then discarded and the pellets processed for small scale plasmid DNA preparation as described in section 2.2.4.

For large scale preparation of M13 mp18 RF DNA, 2.5 ml of an overnight non-competent TG1 cell culture was inoculated with 0.5 ml phage stock and incubated for 5 min at RT. This culture was added to 300 ml of pre-warmed 2 x YT media and incubated for 5 h at 37°C with shaking at 200 rpm. The culture was centrifuged at 20,000 x g for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 50 ml ice-cold STE buffer (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 1 mM EDTA). Cells were pelleted by centrifugation at 20,000 x g for 15 min at 4°C, the supernatant was discarded and a large scale plasmid preparation was carried out.

2.2.14 PREPARATION OF ssM13mp18 DNA

Single stranded M13mp18 DNA for site-directed in-vitro mutagenesis was extracted from the appropriate recombinant bacteriophage. Thus, 50 ml 2 x YT media was inoculated with 500 µl phage stock and 500 µl of a non-competent TG1 cell overnight culture. The culture was incubated at 37°C for 5 h in a shaker at 200 rpm. Phage particles were separated from TG1 cells by centrifugation of 10 x 1 ml samples of culture in 1 ml tubes for 5 min at 12,000 x g. The supernatant from each tube was collected and transferred to a fresh tube. This fraction was centrifuged again for 5 min at 12,000 x g to remove any TG1 cells that would contaminate the resulting DNA preparation with dsDNA. The supernatants were mixed with 200 µl 20 % (w/v) polyethyleneglycol containing 2.5 M NaCl, shaken and incubated at RT for 15 min. Precipitated bacteriophage particles were collected by centrifugation at 12,000 x g for 15 min at RT. The supernatant was discarded,

and centrifugation repeated for 2 min to remove any remaining supernatant from the pellet. The phage particles were resuspended in 100 μ l TE buffer and vortexed for 30 sec. Bacteriophage proteins were removed from the DNA with the addition of 50 μ l Tris-saturated phenol. Samples were vortexed for 20 sec, incubated at RT for 15 min, vortexed for another 20 sec and centrifuged for 5 min at 12,000 x g. The upper layer from each tube was collected and transferred to a new tube. Any contaminating phenol was removed with the addition of 100 μ l of a 24:1 chloroform/amy alcohol mix. Samples were vortexed for 20 sec, then centrifuged at 12,000 x g for 1 min. Supernatants were collected and transferred to new tubes. Ethanol precipitations were carried out on each sample as described in section 2.2.11. However, 25 μ l water was used to dissolve the first ssDNA pellet by warming for 2 min at 37°C and was then transferred to each tube in turn to dissolve all the DNA pellets in the same 25 μ l water. A sample (1 μ l) was analysed by flat-bed gel electrophoresis and viewed under UV illumination to determine the final ssDNA concentration. This method of preparation typically yielded 400 ng ssDNA/ μ l.

2.2.15 AUTOMATED NUCLEOTIDE SEQUENCING

DNA samples were prepared for analysis by automated nucleotide sequencing by mixing 300 ng DNA sample with 3.2 pmol of purified sequencing oligonucleotide primer (designed and purified as described in sections 2.2.16.1 and 2.2.16.2) and water to a final volume of 12 μ l. Further preparation of DNA samples and the automated nucleotide sequencing was performed by Dr. Jurgen Groet (The School of Pharmacy, University of London, U.K.). Briefly, the reaction mixtures were subjected to thermal cycling according to the manufacturers instructions (PE Biosystems, USA). The DNA was precipitated and resuspended in 20 μ l of template suppression reagent. Automated nucleotide sequencing was performed using an ABI PRISM 310 genetic analyser.

2.2.16 OLIGONUCLEOTIDE-DIRECTED *IN-VITRO* MUTAGENESIS

Oligonucleotide-directed *in vitro* mutagenesis was used to generate NR2A and NR2B C-terminal truncation mutants (NR2A^{Trunc} and NR2B^{Trunc}) lacking their ESDV amino acid motifs. The strategy to remove the last four amino acids from the NR2A and NR2B subunits was to convert the codon for glutamate (nucleotide code: GAA [NR2A]; GAG [NR2B]) at amino acid position -3 to a stop codon (TAA or TAG) via a single nucleotide

substitution. The translation of subsequent NR2A and NR2B transcripts would consequently terminate at the -3 amino acid position. Oligonucleotide-directed *in vitro* mutagenesis was also used to introduce a double amino acid substitution in the PSD-95_{c-Myc} N-terminal region. The aim was to introduce two single T→A nucleotide substitutions in the codons for cysteine (TGT) at amino acid positions 3 and 5, resulting in their conversion to codons for serine residues (AGT). The resulting PSD-95_{c-Myc} mutant was termed PSD-95_{c-Myc}(C3S/C5S).

Two mutagenesis kits were used to generate these mutants. They were the Sculptor™ *in vitro* mutagenesis kit for NR2B^{Trunc}, and the Quikchange™ site-directed mutagenesis kit for NR2A^{Trunc} and PSD-95_{c-Myc}(C3S/C5S). Schematic overviews of these two techniques are shown in Figures 2.1 and 2.2 respectively.

The NR2A and NR2B clones for the generation of these mutant constructs were previously subcloned into the pCIS mammalian expression vector (Cik *et al.*, 1993). The PSD-95_{c-Myc} clone was previously subcloned into the pGW1 mammalian expression vector. Schematic maps of these plasmids are shown in Figures 2.3 (pCISNR2A), 2.4 (pCISNR2B) and 2.5 (pGW1PSD-95_{c-Myc}). In addition, maps for the plasmids pCIS and M13mp18 are shown in Figures 2.6 and 2.7 respectively.

2.2.16.1 Design of oligonucleotide primers

Oligonucleotide primers were designed for *in vitro* mutagenesis reactions using the PCRPLAN utility of the PC/GENE software package (Intelligenetics, Inc., California, USA). Sequencing primers were also designed for the confirmation of oligonucleotide mutations by automated nucleotide sequencing using the same software package. A summary of the primers used is shown in Figure 2.8. Parameters for the design of acceptable nucleotide sequences for oligonucleotide primers were as follows:

1. The optimal range of primer length was 15 - 25 nucleotides.
2. The range of acceptable GC percentage was 40 - 60 %.
3. The two bases at the 3' had to be G or C.
4. The maximum level of complementarity to other regions of the template was 70 %.
5. The maximum number of nucleotides forming regions of self-complementarity between separate primers was four.
6. The maximum number of nucleotide pairs forming palindromic sequences resulting in the generation of stem-loop secondary structures within the primer was four.
7. At least 10 nucleotides should be positioned either side of the mismatched mutated nucleotides.

These parameters were taken into account in the design of oligonucleotides for use in both methods of mutagenesis and for use in nucleotide sequencing reactions. However, there were additional features that were important in the design of oligonucleotide primers depending on whether they were to be used in sequencing reactions or in oligonucleotide directed mutagenesis.

Sequencing primers

The melting point of sequencing primers were calculated using the PC/GENE software and were within the acceptable range of 58 - 76°C.

Primers for the use in the Sculptor™ *in vitro* mutagenesis system

A key characteristic of oligonucleotides for use in the Sculptor™ *in vitro* mutagenesis system was that the sequence should not contain a restriction site (target sequence CCGGG) for the enzyme used in the nicking reaction, *Nci* 1. The melting temperature (T_m value) for the primers used in this system were calculated using the PC/GENE software and were within the acceptable range of 58 - 76°C. The T_m value for the NR2B subunit mutagenesis primer was calculated as 43°C which was below the recommended range for oligonucleotide melting points. Elongation of the primer was limited by regions of self complementarity at the 3' end and was limited at the 5' end due to the presence of unknown untranslated sequence beyond the NR2B coding region. The primer sequence was acceptable according to all the other limiting parameters as described in section 2.2.16.1, and was therefore used despite its low melting temperature. The Sculptor™ system of mutagenesis is based on the hybridization of a mutated oligonucleotide primer to single-stranded template DNA. Primers were therefore designed in the minus strand direction.

Primers for the use in the Quikchange™ site-directed method of mutagenesis

The Quikchange method of site-directed mutagenesis is a PCR-based system and thus complementary primers in minus and plus directions were designed. T_m values above 78°C were required for oligonucleotides used in this mutagenesis kit due to the temperature at which primer extension was performed. The T_m values were calculated using the equation shown overleaf.

$$T_m = 81.5 + 0.41(\% \text{ GC}) - \frac{675}{N} - \% \text{ mismatch}$$

% GC	=	% GC content of primer
N	=	Number of nucleotides in primer
% mismatch	=	Percentage of nucleotides not complementary to target strand

2.2.16.2 Purification of oligonucleotide primers

Oligonucleotide primers designed for mutagenesis reactions were purified using Bio-Gel P-30 columns to remove oligonucleotide fragments of less than 20 base pairs, as recommended by the manufacturer (Bio-Rad, Herts, U.K.). This was to prevent the binding of smaller oligonucleotide fragments to non-specific sites on the DNA template. Columns were initially drained of excess buffer by gravity. Buffer exchange was carried out by loading 500 µl TE buffer onto the column followed by centrifugation at 1000 x g at 4 °C. This wash step was repeated twice more and a further round of centrifugation was carried out to remove excess buffer. The oligonucleotide solution (100 µl of 100 pmol/µl) was loaded onto the column and centrifuged at 4°C for 4 min at 1000 x g into a collecting tube. The concentration of oligonucleotide was determined spectrophotometrically.

2.2.16.3 Generation of the NR2B C-terminal truncation mutant

Mutagenesis of the NR2B subunit was carried out using the Sculptor™ *in vitro*-mutagenesis kit. The Sculptor™ mutagenesis system is based on the phosphorothioate technique of Eckstein's group (Nakamaye and Eckstein, 1986) and requires a ssDNA template for the polymerization of mutant DNA from oligonucleotide primers. In order to generate a ssDNA template for the mutagenesis of the NR2B subunit, the entire coding region of the NR2B subunit was subcloned into the M13mp18 bacteriophage vector which carries an origin of replication for ssDNA. This was followed by the purification of ssM13mp18NR2B plasmid DNA for use in the mutagenesis reactions. The mutated NR2B^{Trunc} sequence was then subcloned back into the mammalian expression vector pCIS, for the subsequent transfection and expression in HEK 293 cells.

Subcloning of NR2B into the M13mp18 bacteriophage vector

The NR2B clone was excised from the multiple cloning site of pCISNR2B (shown schematically in Figure 2.4) by restriction enzyme digestion with *EcoRI*, *BamHI* and *ScaI*. The vector contained two *ScaI* restriction sites to yield three vector fragments of approximately 800, 2000 and 2600 nucleotides, aiding separation of the NR2B fragment from the vector by agarose gel electrophoresis. The NR2B fragment was excised and extracted from the agarose gel and purified as described in sections 2.2.9 and 2.2.11. The M13mp18 vector was prepared by restriction enzyme digestion with *EcoRI* and *BamHI* to allow directional cloning of the NR2B fragment into the M13mp18 multiple cloning site. Restricted M13mp18 vector was purified as described in sections 2.2.10 and 2.2.11, and the ligation reaction was performed as described in section 2.2.12. Competent TG1 cells were transformed with the ligation reaction and possible recombinant clones were identified by the blue/white selection procedure as described in detail in section 2.2.13. Five plaques were amplified and dsDNA plasmids prepared. Recombinant clones were identified by an *EcoRI/BamHI/EcoRV* restriction enzyme digestion followed by flat-bed agarose gel electrophoresis. This digest was chosen to excise the 4.5 kb NR2B coding region from the M13 vector (approximately 7.2 kb) at the *EcoRI/BamHI* multiple cloning sites and to digest this NR2B fragment at an *EcoRV* site located at nucleotide position 1389. The results of this restriction enzyme analysis are shown in Figure 2.9. The digestion of clone 3 yielded three restriction fragments of approximately 1.7, 3.1, and 7.4 kb. The sizes of the two smaller restriction fragments were the same as those from the digestion of pCISNR2B (Figure 2.9, lane 7) and the 7 kb fragment was consistent with the size of the M13mp18 vector. The restriction profile of clone 3 therefore confirmed the successful ligation of the NR2B clone into the M13mp18 vector. The NR2B restriction fragments were slightly larger than the size predicted from the NR2B coding sequence due to the presence of untranslated regions at the 5' and 3' ends of the NR2B open reading frame. Single-stranded recombinant M13mp18 NR2B was prepared and purified as described in section 2.2.14.

Phosphorylation of oligonucleotide primers

Oligonucleotide primers for the mutagenesis of the NR2B subunit were designed and purified as described in sections 2.2.16.1 and 2.2.16.2. Oligonucleotides were diluted to a final concentration of 1.6 pmol/ μ l by spectrophotometric quantification at $\lambda = 260$ nm. An OD of 0.025 units/ml per base was equivalent to 1.6 pmol/ μ l. Phosphorylation of oligonucleotides was performed by incubating 30 μ l of oligonucleotide primer solution

with 3 μ l 10 x kinase buffer and 2 units of T4 polynucleotide kinase (30 units/ μ l) at 37°C for 15 min followed by heat inactivation at 70°C for 10 min. The phosphorylated primers were then stored at -20°C or used immediately.

Annealing oligonucleotide to single-stranded DNA template

The annealing step was performed by incubating 2 μ g single-stranded M13mp18NR2B template with phosphorylated oligonucleotide at a molar ratio of oligonucleotide:template of 2:1. The solution was mixed with 1 μ l buffer A (1.4 M MOPS, pH 8, and 1.4 M NaCl) and water to a final volume of 10 μ l. This solution was incubated at 70°C for 3 min, then transferred to a beaker of water at 55°C. The beaker was retained at RT and allowed to cool for 45 min. The reaction was briefly centrifuged to collect the condensate and placed on ice.

Extension and ligation of mutant strand

The annealing reaction was supplemented with 10 μ l deoxynucleoside-5'-triphosphates ([dNTPs]: 1.01 mM deoxy-adenosine-5'-triphosphate [dATP], deoxy-cytidine-5'-[α S]thiotriphosphate [dCTP α S], deoxy-thymosine-5'-triphosphate [dTTP], deoxy-guanosine-5'-triphosphate [dGTP], 2.02 ATP and 20 mM $MgCl_2$), 2.5 units T4 DNA ligase and 0.8 units T7 DNA polymerase. The solution was mixed by flicking, pulse-centrifuged briefly and incubated for 10 min at RT, followed by 30 min at 37°C. The mutant strand was subsequently polymerized from the annealed oligonucleotides and ligated to form a closed circular mutant heteroduplex. The T7 DNA polymerase and T4 DNA ligase were then heat inactivated for 15 min at 70°C.

Removal of single-stranded, non-mutant DNA

The extension reaction was mixed with 50 μ l buffer B (70 mM Tris, pH 8, 10 mM $MgCl_2$, 45 mM NaCl) and 2000 units T5 exonuclease (1000 units/ μ l), a nuclease enzyme specific for single-stranded DNA. This solution was mixed by flicking and incubated at 37°C for 30 min, followed by heat inactivation at 70°C for 15 min.

Nicking of non-mutant strand

The T5 exonuclease digestion was mixed with 5 μ l buffer C (700 mM Tris, pH 8, 350 μ M EDTA, 200 mM DTT) and 5 μ l *Nci* 1 restriction enzyme (5 units/ μ l). The restriction enzyme *Nci* 1, that is unable to cleave phosphorothioated DNA nicks the

non-mutated strand of the heteroduplex leaving the mutant strand intact. The reaction was mixed and incubated at 37°C for 90 min.

Digestion of non-mutant strand

The *Nci I* digestion was mixed with 20 µl buffer D (250 mM Tris pH 8, 150 mM NaCl and 500 µM EDTA) and 160 units exonuclease III. Digestion of the nicked non-mutant strands was carried out at 37°C for 30 min to leave small undigested fragments of non-mutated DNA annealed to intact mutant strands. Exonuclease III was heat inactivated at 70°C for 15 min.

Repolymerization of the mutant strand

The exonuclease III reaction was mixed with 20 µl dNTP mix, 3.5 units DNA polymerase I (3.5 units/µl) and 2.5 units T4 DNA ligase (2.5 units/µl). Repolymerization and ligation of the complementary mutant strand to form a mutant homoduplex was carried out for 1 h at 37°C.

Purification of mutated DNA

The mixture obtained from the final step of the mutagenesis reaction was purified using Qiaex™ DNA resin to remove salts and modifying enzymes, and to concentrate the DNA for more efficient transformation. The sample (80 µl) was divided into two equal aliquots and mixed with 900 µl QX2 buffer (10 mM Tris-HCl, pH 7.0, 8 M NaClO₄) and water up to 300 µl. The Qiaex resin was resuspended by vortexing, then added (10 µl) to each sample and incubated at RT for 10 min. The resin was resuspended in each solution every 2 min to ensure efficient binding to the DNA. The mixture was centrifuged at 12,000 x g for 30 sec at RT and the supernatant discarded. The pellet was washed twice in 500 µl QX3 buffer (10 mM Tris-HCl, pH 7.5, 70 % (v/v) ethanol, 100 mM NaClO₄) and centrifuged at 12,000 x g for 30 sec each time. The final supernatant was discarded and the pellet was allowed to air dry for 10 min. The pellet was resuspended in 20 µl TE buffer by incubation at RT for 5 min with agitation. Following centrifugation at 12,000 x g for 30 sec at RT, the supernatant was collected and transferred to a new tube. The resin was eluted with a further 10 µl TE buffer and combined with the previous eluate.

Identification of mutant clones

Mutated DNA products generated by the Sculptor™ system were amplified using the *E. coli* host strain TG1 due to its higher transformation efficiency with phosphorothioate DNA than other similar strains. Competent TG1 cells were transformed in triplicate with 10 µl purified mutated ssDNA. Potential recombinant plaques were identified using the blue/white selection procedure. Only two plaques were obtained for the mutagenesis of the M13mp18NR2B clone although multiple plaques were obtained from the control reaction. This low efficiency of mutagenesis was perhaps due to the sub-optimal T_m value for the oligonucleotide primer. The two plaques were amplified and double stranded plasmid DNA was prepared. Potential mutant clones were analysed by an *EcoRI/BamHI/EcoRV* restriction enzyme digestion. Both clones displayed the correct restriction profile for M13mp18NR2B^{Trunc} and were therefore analysed by automated nucleotide sequencing as described in section 2.2.15. As shown in Figure 2.10 nucleotide sequencing of the two clones verified that one was M13mp18NR2B^{Trunc}.

Subcloning of NR2B^{Trunc} into the pCIS vector

The NR2B^{Trunc} sequence was digested from the M13 mp18 vector with *EcoRI* and *BamHI*. The restriction enzyme digest was analysed by flat-bed agarose gel electrophoresis and the NR2B^{Trunc} restriction fragment was excised and extracted from the gel. The pCIS vector was prepared by restriction enzyme digestion with *EcoRI* and *BamHI* and was subsequently purified for the ligation reaction. The transformation of competent TOP F' *E. coli* yielded over 50 colonies, 10 of which were selected and processed for the purification of plasmid DNA. Restriction enzyme digestion with *EcoRI*, *BamHI* and *ScaI* (shown in Figure 2.11) verified that all ten clones exhibited the correct restriction profile. Nucleotide sequencing of four clones confirmed these as positive pCISNR2B^{Trunc} recombinants. A glycerol stock of one pCISNR2B^{Trunc} clone was prepared and maxi DNA plasmid preparations performed.

2.2.16.4 Generation of the NR2A C-terminal truncation mutant

The Quikchange™ mutagenesis kit was used to generate the NR2A truncation mutant. The method (illustrated schematically in Figure 2.2) utilised by the Quikchange™ mutagenesis kit does not require ssDNA template for the mutagenesis reaction and was therefore performed directly on the pCISNR2A plasmid. The system utilises *Pfu turbo*

DNA polymerase which has a high fidelity of DNA polymerization compared to other DNA polymerases, and *E.coli* XL1-blue supercompetent cells which possess a higher transformation efficiency than similar strains. This method of mutagenesis was therefore used in place of the Sculptor™ system when it became available.

Annealing oligonucleotides to DNA template and polymerization of mutated DNA

Complementary oligonucleotide primers were designed and purified for the mutagenesis of NR2A. Mutagenesis reactions were prepared by incubating 5 µl 10 x reaction buffer (100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl, pH 8.8, 20 mM MgSO₄, 1 % (v/v) Triton X-100, 1 mg/ml nuclease-free BSA), 5 - 50 ng dsDNA template, 125 ng oligonucleotide primer 1, 125 ng oligonucleotide primer 2, 1 µl dNTP mix, water to a final volume of 50 µl and 1 µl of *Pfu Turbo* DNA polymerase (2.5 U/µl). Three separate reactions were performed using 5 ng, 25 ng, and 50 ng pCISNR2A template while the primer concentration was kept constant. The solutions were subjected to a series of temperature cycles using a PCR Express thermal cycler (Hybaid, U.K.) according to the parameters below. Following temperature cycling the PCR reactions were placed on ice for 2 min and cooled to ≤37°C.

Segment	Cycles	Temperature	Time
1	1	95°C	30 sec
2	14	95°C	30 sec
		55°C	1 min
		68°C	2 min/kb template

Digestion of non-mutated DNA

Non-mutated parent DNA was digested using the endonuclease *Dpn I* which is specific for methylated and hemi-methylated DNA (target sequence Gm6ATC-3'). The template DNA, which was plasmid DNA amplified in *E.coli* is dam methylated and is therefore susceptible to *DpnI* digestion while newly synthesised mutant DNA is not methylated and is therefore resistant to digestion. Thus, 1 µl *DpnI* enzyme (10 U/µl) was added to each reaction and mixed thoroughly by pipetting. The reactions were centrifuged briefly and incubated at 37°C for 1 h.

Transformation of *E.coli* XL1-Blue supercompetent cells

Aliquots (50 µl) of *E.coli* XL1-blue supercompetent cells (provided by the manufacturer) were thawed on ice and transferred to Falcon 2059 polypropylene tubes pre-chilled on ice. The cells were mixed with 1 µl of each *Dpn* 1-treated reaction and incubated on ice for 30 min. The cells were heat-pulsed for 45 sec at 42 °C, returned to ice for 2 min and mixed with 0.5 ml NZY broth (10 g/l NZ amine (casein hydrolysate), 5 g/l yeast extract, 5 g/l NaCl, pH 7.5, containing 12.5 mM MgCl₂, 12.5 mM MgSO₄, and 0.4 % (w/v) glucose, filter-sterilized and pre-heated to 42°C). Transformation reactions were incubated at 37°C for 1 h with shaking at 225 rpm. The entire volume of cells was finally plated onto LB agar plates containing 50 µg/ml ampicillin and incubated for 15 - 20 h at 37°C. Over 100 potential mutant pCISNR2A clones were obtained from each transformation. Plasmid DNA was isolated from 10 selected colonies and screened by an *EcoRI* restriction enzyme digestion. The restriction enzyme *EcoRI* was used to excise the NR2A coding region from the multiple cloning site of pCIS in order to verify the size of the insert as approximately 4.4 kb and the size of the vector as approximately 5.4 kb. Six clones exhibited the correct restriction profile and were analysed by automated nucleotide sequencing to screen for positive mutants. All six clones contained the substituted nucleotide and were thus pCISNR2A^{Trunc}. The sequencing data for one of these clones is shown in Figure 2.12. Competent TOP 10 F' *E.coli* were then transformed with pCISNR2A^{Trunc} for the preparation of bacterial glycerol stocks and for maxi DNA plasmid preparation.

2.2.16.5 Generation of the PSD-95_{c-Myc}(C3S/C5S) mutant

The mutagenesis of PSD-95_{c-Myc} was performed using the QuikchangeTM mutagenesis kit. Complementary oligonucleotide primers were designed and purified, and mutagenesis reactions were carried out as described in section 2.2.16.4. Following the transformation of XL1-blue supercompetent *E.coli* with each mutagenesis reaction, over 100 colonies were obtained, ten of which were selected and processed for the purification of plasmid DNA. A preliminary screen was performed by restriction enzyme digestion with *EcoRV* which digests at a restriction site located in the multiple cloning site of the pGW1 vector at the 3' end of the PSD-95_{c-Myc} coding region and in the sequence encoding the c-Myc tag of PSD-95_{c-Myc} at nucleotide position 50. This restriction enzyme digestion of pGW1PSD-95_{c-Myc} yielded a vector fragment of approximately 5 kb and a PSD-95_{c-Myc}

fragment of approximately 2.1 kb. Analysis of the resulting restriction enzyme digests by flat bed gel electrophoresis revealed that all ten clones exhibited the correct restriction profile. The analysis of two clones by automated nucleotide sequencing confirmed that both carried the desired mutation. The sequencing data for one of these clones is shown in Figure 2.12. Competent TOP 10 F' *E.coli* were subsequently transformed with pGW1PSD-95_{c-Myc}(C3S/C5S) for the preparation of bacterial glycerol stocks and for maxi DNA plasmid preparation.

2.2.17 PREPARATION OF CELL CULTURE MEDIA

Growth media for the routine culturing of HEK 293 cells was prepared by dissolving 15.6 g powdered Dulbecco's modified Eagles medium nutrient mixture F-12 HAM (DMEM/F-12) in approximately 100 ml sterile water. This was supplemented with 100 ml fetal calf serum (FCS), 20 ml penicillin/streptomycin (PenStrep, 10,000 units/ml), 40 ml 7.5 % (w/v) NaHCO₃ and sterile water up to a final volume of 1 l. The media was adjusted to pH 7.6 with 10 M NaOH, then filter-sterilised using a 0.2 µm Sartorius Sartolab-V150 filter unit and stored at 4°C.

During the transfection procedure it was necessary to culture cells in L-glutamine-free media. Growth media was therefore prepared from liquid L-glutamine-free DMEM/F-12 media which was mixed with 100 ml FCS, 24 ml 7.5 % (w/v) NaHCO₃, and 20 ml PenStrep (10,000 units/ml). A final volume of 1 l was achieved with the addition of liquid L-glutamine-free DMEM/F-12. Again the media was adjusted to a pH of 7.6 with 10 M NaOH, then filter-sterilised and stored at 4°C.

2.2.18 HUMAN EMBRYONIC KIDNEY 293 CELL CULTURE

HEK 293 cells were routinely cultured under sterile conditions in 250 ml Cellstar sterile culture flasks in an IR 1500 automatic CO₂ Incubator (Flow Laboratories, England) at 37°C and at a CO₂ concentration of 5 %. It was necessary to subculture the cells after growth had been maintained for 3 - 4 days or to a confluency of 70 - 80 %. All solutions used in this procedure were pre-warmed to 37°C. Subculturing was carried out by removing the growth media and rinsing the cells in 5 ml Hank's balanced salt solution. The cells were incubated with 2 ml 1 x Trypsin-EDTA for 1 min at 37°C, then gently resuspended in 10 ml DMEM/F-12 growth media using a hand-held aspirator. Finally, the cell suspension

was divided into 3 ml aliquots in separate culture flasks and mixed with 9 ml DMEM/F-12 growth media.

2.2.19 LIQUID NITROGEN STORAGE OF HEK 293 CELLS

After a period of 2 passages, stocks of HEK 293 cells were prepared and stored in liquid nitrogen for future use. HEK 293 cells were cultured to a confluency of approximately 70 % and trypsinized as described in section 2.2.18 except the cells were finally resuspended in 50 ml DMEM/F-12 growth media and decanted into a sterile 50 ml falcon tube. Cells were collected by centrifugation at 3,700 x g for 5 min at RT. The supernatant was removed and the pellet resuspended in 6 ml cryoprotective media (4.8 ml DMEM/F-12 growth media, 0.6 ml FCS and 0.6 ml dimethyl sulphoxide [DMSO]). This cell preparation was subdivided into 2 ml aliquots and stored in sterile cryogenic vials at -80°C for 24 h. The tubes were finally transferred to liquid nitrogen for long-term storage.

2.2.20 REVIVAL OF HEK 293 CELLS FROM LIQUID NITROGEN STORAGE

HEK 293 cells were routinely cultured for up to 3 months or for a total of 25 - 30 passages. Subjecting HEK 293 cells to a greater number of passages resulted in a decrease in the efficiency of transfection with mammalian expression vectors (determined as described in section 2.2.23). After 25 - 30 passages a fresh aliquot of cells was revived from liquid nitrogen storage. A vial of HEK 293 cells was thawed rapidly at 37°C and decanted into 50 ml DMEM/F-12 pre-warmed to 37°C. Cells were pelleted by centrifugation at 3,700 x g for 1 min at RT, resuspended in 12 ml DMEM/F-12 growth media and finally transferred to a sterile culture flask.

2.2.21 TRANSIENT EXPRESSION OF NMDA RECEPTORS IN HEK 293 CELLS

The calcium phosphate-mediated transfection of HEK 293 cells

Transfection of HEK 293 cells by the calcium phosphate method was carried out according to the method of Gorman *et al.* (1990). HEK 293 cells were grown to approximately 90 % confluency (3 - 4 x 10⁶ cells per flask) 24 h prior to transfection and subcultured as described in section 2.2.9. The growth media was replaced with

L-glutamine-free DMEM/F-12 media 3 h before transfection and the cells were incubated at 37°C and at an increased CO₂ concentration of 7.5 %. DNA samples were prepared 30 min prior to transfection. A maximum of 20 µg plasmid DNA was diluted in $\frac{1}{10} \times$ TE buffer to a final volume of 450 µl in an Eppendorf tube and allowed to equilibrate to RT. In a separate tube, 500 µl 2 x HEPES buffered saline (50 mM N-2hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), pH 7.12, 280 mM NaCl and 1.1 mM Na₂HPO₄, [2 x HBS]) was also allowed to equilibrate to RT. Transfections were performed by pipetting 50 µl 2.5 M CaCl₂, pre-warmed to 37 °C, dropwise into the plasmid DNA solution with vigorous shaking by hand for 15 sec. This solution was pipetted into the 2 x HBS solution dropwise at a rate of approximately 50 µl/5 sec and mixed by pipetting up and down 10 times using a 2 ml pipette. The resulting calcium phosphate precipitate was pipetted dropwise evenly over the HEK 293 cells and incubated at 37°C for 3 h at a CO₂ concentration of 7.5 %. Glycerol shocking was then carried out to increase the efficiency of transfection. The cell culture media was removed by aspiration and the cells were incubated with 1.5 ml 15 % (v/v) glycerol in phosphate buffered saline ([PBS], 4 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4, 137 mM NaCl and 1.7 mM KCl) for 30 sec at RT. The glycerol/PBS solution was removed by aspiration and the cells were rinsed in 5 ml L-glutamine-free DMEM/F-12 media by gentle rocking. Finally, the cells were bathed in 10 ml L-glutamine-free DMEM/F-12 media and incubated at 37°C for 16 - 40 h post-glycerol shock at a CO₂ concentration of 5 %. The expression of functional NMDA receptors in HEK 293 cells results in cell death post-transfection due to the permeability of NMDA receptors to cations, in particular calcium (Cik *et al.*, 1993). Cells co-transfected with NR1 and NR2 subunits therefore required the addition, post-glycerol shock, of the NMDA receptor antagonist, ketamine, to a final concentration of 1 mM to prevent NMDA receptor-mediated cell death.

Transfection of HEK 293 cells with NMDA receptor clones

The plasmids used for the transient expression of NMDA receptors in HEK 293 cells were:

NR1 subunit clones (mouse):	pCISNMDAR1-1a, pCISNMDAR1-2a and pCISNMDAR1-4b
NR2 subunit clones (rat):	pCISNMDAR2A and pCISNMDAR2B
PSD-95 clones (rat):	pGW1PSD-95 and pGW1PSD-95 _{c-Myc}
Control plasmids:	pSV-β-galactosidase and pCIS
Others:	pGFP-C1

For the expression of single NMDA receptor subunits, 10 µg plasmid DNA was used. The co-expression of NR2 and NR1 subunits used a plasmid ratio of 3:1 which resulted in the optimal expression of functional NMDA receptors (Cik *et al.*, 1993). For the co-expression of NMDA receptors with PSD-95, a 1:1 ratio of NMDA receptor:PSD-95 clones was used. Thus, a triple transfection used 2.5 µg pCISNR1, 7.5 µg pCISNR2 and 10 µg pGW1PSD-95 or pGW1PSD-95_{c-Myc}. For transfections performed in parallel in the absence of PSD-95, 10 µg of either pSV-β-galactosidase or pCIS were included as controls to achieve a final mass of 20 µg plasmid DNA.

2.2.22 PREPARATION OF TRANSFECTED HEK 293 CELLS FOR ELECTROPHYSIOLOGICAL STUDIES

HEK 293 cells prepared for electrophysiological studies were cultured on 13 mm diameter glass coverslips. The coverslips were pre-coated with poly-L-lysine to aid adhesion of the cells to the glass surface. Thus, three coverslips (diameter 13 mm) were placed in a Petri dish (diameter 35 mm) and incubated with 3 ml sterile poly-L-lysine (50 µg/ml) for 1 h at RT. The coverslips were rinsed in 4 x 3 ml sterile water and allowed to dry under sterile conditions. HEK 293 cells were transfected with NMDA receptor clones in the presence of 4 µg pGFP-C1 plasmid using the calcium phosphate transfection method as described in section 2.2.21. The pGFP-C1 plasmid encodes green fluorescent protein (GFP) and enabled the identification of transfected cells under illumination with UV light. Transfected HEK 293 cells were cultured for 1 h post-glycerol shock, then trypsinized as described in section 2.2.18. The cells were resuspended with the addition of 9 ml L-glutamine free DMEM/F-12 media, pre-warmed to 37°C. Two Petri dishes, each containing three coverslips, were prepared per transfected HEK 293 cell sample in order to generate two cultures of differing cell density, one containing 1.5 ml of resuspended cells and the other containing 0.75 ml of resuspended cells. L-glutamine free media was added to each dish to achieve a final volume of 3 ml. The cells were cultured for 16 h post-trypsinization in the presence of 200 µM AP5 for the prevention of NMDA receptor mediated cell death.

2.2.23 ASSESSING TRANSFECTION EFFICIENCY USING THE CYTOTOX 96™ NON-RADIOACTIVE CYTOTOXICITY ASSAY

The percentage cell death following the co-expression of NR1 and NR2 subunits in HEK 293 cells represents the percentage transfection efficiency. This can be assayed by the measurement of a stable cytosolic enzyme, lactate dehydrogenase (LDH), released upon cell lysis. The Cytotox96™ kit quantitatively measures the activity of LDH via the conversion of tetrazolium salt into a red formazan product. The protocol supplied by the manufacturer (Promega, U.K.) was modified to measure LDH activity released from HEK 293 cells following transfection with NMDA receptor clones. Thus, HEK 293 cells were transfected and cultured in the absence of ketamine for 16 h post-transfection. A sample (1 ml) of media (sample A) was removed and centrifuged at 12,000 x g for 5 min at 4°C. The supernatant was collected, diluted 1:3 and 1:10 in Hanks balanced salt solution and retained at RT. To assay for the total LDH activity, the flask of cells was incubated at -80°C for 30 min to induce complete cell lysis. The cells were thawed at 37°C for 15 min and a 1 ml sample of media (sample B) collected. Cellular debris was pelleted by centrifugation at 12,000 x g for 5 min at 4°C. The supernatant was collected, diluted 1:10 and 1:20 in Hanks balanced salt solution and retained at RT. Triplicate samples (50 µl) of each dilution were loaded onto a 96 well plate and 50 µl reconstituted substrate mix was added to each well. Blank samples were assayed in parallel using 50 µl Hanks balanced salt solution. The reactions were incubated for 30 min under aluminium foil, then terminated with the addition of 50 µl stop solution. The $OD_{\lambda=490nm}$ was measured for each well using a Dynatech minireader. Absorbance values were adjusted according to their initial sample dilution and the percentage LDH activity was calculated using the following equation.

$$\frac{\text{LDH activity in sample A}}{\text{LDH activity in sample B}} \times 100$$

To ensure the accurate determination of percentage transfection efficiency the value calculated using the above method was adjusted for background LDH activity in the culture media due to spontaneous cell death. Percentage background LDH was assayed by measuring LDH activity in the media from transfected HEK 293 cells cultured in the presence of 1 mM ketamine using the above method. Transfection efficiencies of 25-30 % were routinely achieved. Background levels of LDH activity were typically 3-5 %.

2.2.24 PREPARATION OF HEK 293 CELL HOMOGENATES

Transfected HEK 293 cells were prepared for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, see section 2.2.27) and radioligand binding (see section 2.2.30) according to the following method unless stated otherwise. HEK 293 cells were transfected as described and cultured for 16 - 40 h post-transfection. Cells were harvested from the flask interior using a sterile cell scraper and transferred to a JA20 centrifuge tube. Following centrifugation at 1,000 x g for 5 min at 4°C, the supernatant was discarded. The cell pellet was then homogenised (20 strokes) in 3 - 4 ml of ice-cold Tris-citrate buffer (50 mM Tris-citrate, pH 7.1 at 4°C, 5 mM EDTA and 5 mM EGTA) using a tight 7 ml Wheaton Dounce glass-glass homogeniser. The homogenate was returned to the centrifuge tube and Tris-citrate buffer was added to a final volume of approximately 30 ml. The cell homogenate was pelleted by centrifugation at 27,000 x g for 30 min at 4°C and the supernatant was discarded. Homogenisation and centrifugation was repeated and the pellet was finally resuspended in Tris-citrate buffer by homogenisation to yield a concentration of approximately 0.5 mg protein/ml. A single transfected flask typically yielded 1 mg protein. For long-term storage the cell homogenate was aliquoted into appropriate volumes and stored at -20°C.

2.2.25 PREPARATION OF RAT FOREBRAIN MEMBRANES

Adult rats, Wistar strain, were rapidly stunned and decapitated. Whole brains were removed and placed on a dissection plate at 4°C. A crude dissection was performed to separate the cerebellum from the forebrain and to remove and discard the majority of the white matter. The resulting brain tissue was snap frozen in liquid nitrogen and subsequently stored at -20 °C until further processing was carried out. The P2 membrane fraction was prepared from the forebrain tissue using the method described by Chazot *et al.* (1993). Brains were initially weighed then slowly thawed in 50 ml of ice-cold sucrose buffer (50 mM Tris-citrate, pH 7.4 at 4 °C, 5 mM EDTA, 5 mM EGTA, 320 mM sucrose, supplemented with 1 mg/ml trypsin inhibitor type II-S, trypsin inhibitor type II-O, benzamidine, and bacitracin). Forebrain tissue was crudely fragmented using a spatula and sucrose buffer was added to a final volume of approximately 120 ml. Phenylmethyl sulphonyl fluoride (PMSF) was added to a final concentration of 1 mM immediately prior to homogenisation using a Potter S glass-teflon homogeniser (B. Braun, Biotech

International) at 850-900 rpm, on ice, for 10 - 15 strokes. The brain homogenate was decanted into a JA14 centrifuge tube and a final volume of 150 ml was obtained with the further addition of sucrose buffer. Centrifugation at 800 x g for 10 min at 4°C was carried out and the upper layer was collected and retained on ice. The P1 pellet was resuspended to a final volume of 120 ml in sucrose buffer, then homogenised and centrifuged again. The upper layer was collected and pooled with the supernatant from the previous round of centrifugation. This fraction was centrifuged at 30,000 x g for 30 min at 4°C to yield the P2 pellet. The supernatant was decanted and discarded, and the pellet was resuspended in approximately 50 ml of ice-cold Tris-citrate buffer by homogenisation to achieve a smooth P2 homogenate. This was frozen at -20°C. A series of freeze-thaw wash cycles were carried out on the homogenate to remove endogenous NMDA receptor ligands from solution, in particular glutamate and glycine. Thus, the homogenate was thawed slowly to 4°C, diluted to a final volume of 500 ml with Tris -citrate buffer and subjected to centrifugation at 27,000 x g for 30 min at 4°C. The resulting pellet was resuspended in approximately 50 ml Tris-citrate buffer and snap frozen in liquid nitrogen. This cycle was repeated 5 times and following the final round of centrifugation the pellet was resuspended in Tris-citrate buffer to a concentration of 25 % (w/v). Aliquots (1 ml) of the resulting forebrain membrane preparation were stored at -20°C for up to 6 months with no detectable loss of [³H]MK801 specific ligand binding activity.

2.2.26 PROTEIN QUANTIFICATION USING THE LOWRY ASSAY

Protein concentrations were determined using the method of Lowry *et al.*, (1951).

Three stock solutions were prepared prior to performing the Lowry assay:

Reagent A: 2 % (w/v) Na₂CO₃, 0.1 M NaOH, 0.5 % (w/v) SDS

Reagent B: 2 % (w/v) sodium potassium tartrate (KNaC₄H₄O₆)

Reagent C: 1 % (w/v) CuSO₄

Assay reagent: 100 parts reagent A: 1 part reagent B and 1 part reagent C

The optimal quantity of protein for accurate determination by the Lowry assay was 30-60 µg. Therefore, an aliquot of HEK 293 cell homogenate or rat forebrain P2 membrane preparation estimated to contain approximately 50 µg was selected and diluted in 1 ml 10 % (w/v) trichloroacetic acid (TCA) in triplicate. The samples were vortexed and

centrifuged for 5 min at 12,000 x g in a microcentrifuge. The supernatant was removed and discarded, and the pellets allowed to air dry for 10 min. Protein standards in the range 0 - 100 µg protein were prepared in triplicate using bovine albumin serum (BSA) dissolved in 100 µl water. The dried protein pellets and protein standards were mixed with 0.5 ml assay reagent. The mixtures were incubated for 10 min at RT and repeatedly vortexed to dissolve the pellets. Folin-Ciocalteu phenol reagent was diluted 1:1 in water and 50 µl was added to each sample. The solutions were immediately vortexed and incubated for 30 min at RT. Each sample was diluted with 0.5 ml water and the $OD_{\lambda=750nm}$ measured using a spectrophotometer. The protein concentration was determined using the BSA standard calibration curve.

2.2.27 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Preparation of 7 % polyacrylamide resolving gels

Transfected HEK 293 cell homogenates were analysed by SDS-PAGE using 7 % polyacrylamide resolving gels. The gels were prepared by mixing 31 ml water with 15 ml gel running buffer (1.5 M Tris, pH 8.8, 8 mM EDTA and 0.4 % SDS), 14 ml 30 % (w/v) acrylamide solution, and 30 µl *N, N, N', N'*-Tetramethylethylenediamine (TEMED). The solution was degassed for 30 min under vacuum. Polymerization was initiated by the addition of 300 µl 10 % (w/v) ammonium persulphate (APS) and the solution was poured into a gel tray containing 11 assembled vertical slab gel plates. Gels were overlaid with hydrated butan-1-ol to a depth of approximately 2 mm to ensure the acrylamide gels polymerized with a flat surface. Gels were allowed to polymerize at RT for 1 hr, then stored at 4°C in gel running buffer diluted 1:4 in water.

Preparation of the stacking gel

The stacking gel was prepared by mixing 2.3 ml water, 1 ml stacking gel buffer (0.5 M Tris, pH 6.8, 8 mM EDTA and 0.4 % (w/v) SDS), 0.65 ml 30 % (w/v) acrylamide solution, and 5 µl TEMED, then degassed under vacuum for 15 min. Polymerization was initiated by the addition of 80 µl 10 % (w/v) APS and the solution was immediately pipetted onto the top of the resolving gel. The well comb was inserted and the gel allowed to polymerize for 10 min.

Sample preparation

Precipitation of proteins from transfected HEK 293 cell homogenates was performed using the methanol/chloroform method of protein precipitation. HEK 293 cells homogenates were prepared as described in section 2.2.24. Aliquots of cell homogenate known to contain approximately 25 µg of protein were diluted in Tris-citrate buffer to a final volume of 100 µl and mixed with 400 µl methanol. The sample was vortexed and subjected to a pulse of centrifugation up to 12,000 x g. Chloroform (100 µl) was added and the sample was vortexed and centrifuged again. Water (300 µl) was added and the sample vortexed and centrifuged for 1 min at 12,000 x g. The upper aqueous phase was collected leaving the protein at the interface between the aqueous and organic phases. Methanol (100 µl) was then added and mixed by flicking. The resulting protein precipitate was pelleted by centrifugation at 12,000 x g for 4 min, the supernatant was removed and the pellet dried under vacuum. The protein pellet was solubilized in 5 µl 3 x sample buffer (30 mM NaH₂PO₄, pH 7.0, 30 % (v/v) glycerol, 0.05 % (v/v) bromophenol blue and 7.5 % (w/v) SDS), 8.5 µl water and 1.5 µl 1 M DTT, and finally boiled for 5 min in a water bath. Alternatively samples were stored at -20°C, without the addition of DTT, for later use. Protein standards were purchased as dried powder and were resuspended in 3 x sample buffer. Protein standards were prepared for SDS-PAGE by mixing 2 µl resuspended standard, 1.5 µl 1 M DTT and 6.5 µl water.

Gel loading

Gel plates were assembled in a Mighty Small II SE260 electrophoresis unit and bathed in electrophoresis buffer (50 mM Tris, pH 8.8, 384 mM glycine, 1.8 mM EDTA and 1.8 % (w/v) SDS). Samples were prepared as described above and were loaded immediately after boiling using a Hamilton microlitre syringe. Electrophoresis was carried out at 10 mAmp constant current for an initial 10 min, followed by 15 mAmp constant current until the bromophenol blue band migrated to the bottom of the gel.

2.2.28 IMMUNOBLOTTING

Electrophoretic transfer of proteins to nitrocellulose

Electrophoretic transfer of proteins from the SDS-polyacrylamide gel to 0.1 µm nitrocellulose (Protran BA 79 Cellulosenitrate) was performed using the Hoefer Transfer Electrophoresis Unit. The nitrocellulose membrane was pre-equilibrated for 20 min in

transfer buffer (25 mM Tris, pH 8.5, 192 mM glycine and 20 % (v/v) methanol). The gel plate was disassembled and the gel was rinsed briefly in water. A transfer cassette was assembled from sheets of foam and blotting paper, briefly soaked in transfer buffer. Thus, the 'sandwich' consisted of a layer of foam, two sheets of blotting paper, the nitrocellulose membrane, the polyacrylamide gel, two more sheets of blotting paper and another layer of foam. Any air bubbles trapped between layers were removed by rolling a Pasteur pipette over the paper layers. The transfer cassette was submerged in the transfer buffer of the electrophoresis unit, and electrophoresis was carried out at 50 V constant voltage for 2.5 h. The transfer cassette was then disassembled and the nitrocellulose trimmed to the shape of the gel. The nitrocellulose was finally rinsed superficially in phosphate buffered saline ([PBS] 136.9 mM NaCl, 2.68 mM KCl, 10.1 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) and either processed for the detection of immune products or stored at 4°C in PBS.

Blocking the nitrocellulose membrane

Non-specific binding sites on the nitrocellulose membrane were blocked by an initial overnight incubation in 10 ml of blocking buffer (PBS supplemented with 5 % (w/v) 99 % fat free milk powder and 0.2 % (v/v) polyoxyethylene sorbitan monolaurate [TWEEN-20]) at 4 °C with gentle shaking.

Incubating with primary antibody

The nitrocellulose was rinsed in PBS and incubated with the primary antibody diluted in an appropriate volume of 2.5 % (w/v) milk powder in PBS for 1 hr at RT with gentle shaking. The antibody solution was removed and the nitrocellulose washed 4 times in 15 ml wash buffer (PBS containing 2.5 % (w/v) milk and 0.2 % (w/v) TWEEN-20) for 10 - 15 min at RT with gentle shaking.

Polyclonal anti-PSD-95 antibodies were raised in rabbits against the peptide CSKRRVERRE (490-498) by Dr. P. L. Chazot. Wild-type PSD-95 or PSD-95_{c-Myc} proteins were detected by immunoblotting using either the anti-rat PSD-95 (480-498) antibody at a concentration of 2-5 µg/ml or with commercial anti-c-Myc (9E10) mouse monoclonal antibodies diluted 1:1000. The detection of NMDA receptor subunit proteins by immunoblotting was performed with the use of immunoaffinity-purified subunit specific polyclonal antibodies at final concentrations of 0.5 - 1 µg/ml. The following peptide-specific antibodies (listed overleaf) were raised in rabbits and previously characterised as indicated.

1.	anti-rat NR1 (911-920)	Chazot <i>et al.</i> 1992)
2.	anti-rat NR1 (17-35)	(Chazot and Stephenson, 1997a)
3.	anti-rat NR2A (1,381-1,394)	(Hawkins <i>et al.</i> 1999)
4.	anti-rat NR2A/2B (1,435-1,445)	(Cik <i>et al.</i> 1993)
5.	anti-rat NR2B (46-60)	(Chazot and Stephenson, 1997b).

Incubating with secondary antibody

Either anti-rabbit immunoglobulin HRP-linked whole antibody (from donkey) or anti-mouse immunoglobulin HRP-linked whole antibody (from sheep) were diluted 1:2000 in PBS supplemented with 2.5 % (w/v) milk powder. The nitrocellulose membrane was incubated with the appropriate secondary antibody for 1 h at RT with gentle shaking, then washed 4 times in 15 ml wash buffer for 10 - 15 min at RT with gentle shaking. The membrane was finally rinsed in PBS for 5 min at RT with gentle shaking.

Detection of HRP-linked antibodies

Detection of bound HRP-linked secondary antibodies was performed using the ECL^{Plus} kit which contains a chemiluminescent agent activated by the peroxidase mediated catalytic breakdown of H₂O₂. The procedure was carried out as recommended by the manufacturer (Amersham Pharmacia, Bucks. U.K.). Briefly, the nitrocellulose was drained of PBS or TBS and incubated in a 1:1 (v/v) mixture of Solution 1 and Solution 2 for 60 sec. The membrane was drained of excess solution, wrapped in clingfilm and transferred to a developing cassette. Fluorescent immunoreactive bands were detected by an initial 30 sec exposure to high performance chemiluminescence film (HyperfilmTM ECLTM). Subsequent exposures were timed according to the intensity of the initial signals obtained. The Hyperfilm was developed by submersion in Kodak GBX developer for 2 minutes, followed by a brief rinse in water and fixation in Kodak GBX fixer solution for at least 2 min. In order to perform a second or third immunoblot on the same membrane, the membrane was removed from the clingfilm and washed in PBS for 2 min. The immunoblotting procedure was then repeated.

Quantification and comparison of immunoreactive band intensities

Immunoreactive bands were scanned using a Molecular Dynamics Personal Densitometer and analysed by optical densitometry using the ImageQuant software

package. When comparing the relative expression of a recombinant protein in two separately transfected HEK 293 cell samples, a number of steps were taken to ensure an accurate quantitative comparison was being made. Transfections of HEK 293 cells with the plasmid cDNA clones were performed in parallel on separate flasks that were subcultured from the same original flask to ensure equal cell density between the two samples. Transfections were performed in triplicate and the three flasks from each triplicate transfection were pooled during cell harvesting to minimize variations in transfection efficiency. Equal quantities (verified by protein quantification) of cell homogenate from each sample were applied per gel lane in triplicate on the same SDS-polyacrylamide gel to minimize variations in loading between separate lanes. Fold differences in the optical density of immunoreactive bands obtained between the two triplicate samples were averaged between approximately three exposures of the same immunoblot. Detection of immunoreactive bands by ECLTM was performed in the linear range of the film.

Detection of phosphoserine, phosphothreonine and phosphotyrosine residues by immunoblotting

The immunoblotting procedure was modified for the use of rabbit anti-phosphoserine, rabbit anti-phosphothreonine, and mouse anti-phosphotyrosine antibodies due to the presence of phosphorylated proteins in milk powder. Non-specific binding to the nitrocellulose membrane was initially blocked by an overnight incubation at 4°C with gentle shaking in Tris buffered saline ([TBS], 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl) containing 5 % (w/v) BSA and 0.1 % (v/v) TWEEN-20. Primary antibodies were diluted to a final concentration of 0.5 µg/ml in TBS containing 3 % (w/v) BSA and 0.1 % (v/v) TWEEN-20 and incubations were carried out for 1 h at RT. The membrane was subjected to 4 x 25 min washes in TBS containing 0.1 % (v/v) TWEEN-20. Incubations with appropriate secondary antibodies diluted 1:2000 in TBS containing 3 % (w/v) BSA and 0.1 % (v/v) TWEEN-20 were carried out for 1 h at RT followed by 4 x 25 min washes in TBS containing 0.1 % (v/v) TWEEN-20. The membrane was finally rinsed in TBS for 5 min. The specificity of the anti-phosphotyrosine antibody for phosphorylated tyrosine residues was determined by pre-incubation of the antibody, diluted 0.5 µg/ml in TBS (containing 3 % (w/v) BSA and 0.1 % (v/v) TWEEN-20), with 20 mM phosphotyrosine for 10 min prior to application to the membrane.

2.2.29 IMMUNOPRECIPITATION ASSAYS

The isolation of specific recombinant proteins expressed in transfected HEK 293 cells was performed using the method of immunoprecipitation as described by Chazot *et al.* (1994). HEK 293 cells were transfected and cultured for 16 h post-transfection. Cells were harvested and homogenised as described in section 2.2.24 except that following one round of centrifugation at 27,000 x g, the pellet was detergent solubilized as described in the next section. Typically 4 mg cell protein provided suitable volumes to perform the immunoprecipitation assay.

Solubilization of integral membrane proteins from HEK 293 Cells

The pellet of transfected HEK 293 cell homogenate was resuspended in ice-cold solubilization buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 % (v/v) Triton X-100 supplemented with trypsin inhibitor type II-S, trypsin inhibitor type II-O, benzamidine, and bacitracin to a final concentration of 5 µg/ml) by homogenisation (10 strokes) using a tight 7 ml Wheaton Dounce glass-glass homogeniser to a final concentration of approximately 1.5 mg protein/ml. PMSF was added to a final concentration of 0.5 mM just prior to the homogenisation step. The resuspended cell homogenate was transferred to a 50 ml falcon tube and incubated for 1 h at 4°C with gentle shaking. A final concentration of 1mg protein/ml of the HEK 293 cell lysate was then achieved by the further addition of solubilization buffer. Insoluble cellular debris was pelleted by centrifugation at 100,000 x g for 30 min at 4°C using a Beckman Optima™ MAX-E Ultracentrifuge. The resulting supernatant was collected and any fragments of insoluble material remaining in the soluble fraction were removed by filtration using a 0.2 µm syringe filter. Aliquots of solubilized protein (3 x 50 µl) were collected and stored at -20 °C for future analysis by SDS-PAGE. The insoluble pellet was resuspended in an equivalent volume of Tris-citrate buffer by vigorous homogenisation with a tight 7 ml Wheaton Dounce glass-glass homogeniser until a smooth suspension was obtained. Samples (50 µl) were then stored at -20 °C until future use.

Immunoprecipitation reactions

The initial step of the immunoprecipitation assay was performed by incubating 300 µl detergent solubilized protein (as described above) with 5 µg antibody at RT for 1 hr with gentle agitation. Antibody/antigen complexes were precipitated by a 1 h

incubation at 4°C with 5 mg protein A sepharose (protein A immobilized on cyanogen bromide-activated sepharose, re-hydrated in PBS to a concentration of 250 mg/ml). Immune complexes were then collected by centrifugation at 3000 x g for 15 sec. The supernatant was removed carefully using a Gilson pipette and retained for future analysis. The sepharose pellet was washed in 1 ml solubilization buffer and centrifugation was repeated. A further cycle of washing with 1 ml solubilization buffer was performed. After the final centrifugation the remaining supernatant was removed using a Hamilton microlitre syringe. Protein A-bound antibodies and antigens were eluted in 5 µl 3 x sample buffer and 8.5 µl water, and stored at -20°C. Control reactions to confirm specific binding of the antibodies were performed in parallel using 5 µl rabbit non-immune immunoglobulins (purified by Dr. K. Brickley). For the analysis of immune pellets by SDS-PAGE, samples were thawed and vortexed with 1.5 µl 1 M DTT. Samples were boiled for 5 min in a water bath, vortexed and pulse-centrifuged, then analysed by SDS-PAGE as described in section 2.2.27.

Investigation of the phosphorylation state of immunoprecipitated proteins

The immunoprecipitation assay was modified slightly when immune pellets were to be analysed by immunoblotting using anti-phosphorylated residue antibodies as described in section 2.2.28. During the preparation of the HEK 293 cell membrane fraction and during the solubilization procedure, protein phosphatase substrates and inhibitors were included in both the Tris-citrate buffer and the solubilization buffer to prevent dephosphorylation of phosphorylated proteins. Thus, both buffers were supplemented with 1 mM Na₃VO₄, 30 mM sodium pyrophosphate (Na₄P₂O₇) and 50 mM NaF.

2.2.30 RADIOLIGAND BINDING ASSAYS

2.2.30.1 The method of polyethylenimine filtration

Radioligand binding assays were performed on HEK 293 cell homogenates and rat forebrain preparations using the method of polyethylenimine filtration. Routinely, 50 µg protein was incubated in triplicate with an appropriate dilution of tritiated radioligand until equilibrium was reached. Bound radioligand was separated from unbound ligand by rapid filtration using a Brandel M-24R cell harvester through Whatman GF/B glass microfiber filters pre-soaked in 1 % (v/v) polyethylenimine for 30 min at 4°C. Filters were washed three times with 4 ml wash buffer (10 mM NaH₂PO₄, pH 7.4 with 2M KOH)

at 4°C. The filter discs were then transferred to mini-vials containing 4 ml Optiphase safe scintillation fluid and incubated at RT for 36 - 48 h. Vials were vortexed and bound radioactivity was measured using a Beckman LS 5000CE scintillation counter with a counting time of 3 min per vial.

2.2.30.2 [³H]MK801 radioligand binding assays

Routinely, 50 µg protein was incubated in triplicate with 10 µM or 1 mM L-glutamate (as indicated), 30 µM glycine and [³H]MK801 in Tris-citrate buffer to a final volume of 200 µl. Non-specific binding was determined using 100 µM TCP. Incubations were carried out for 2 h at RT and terminated using the method of polyethylenimine filtration as described above (Chazot *et al.*, 1994). Competitive binding studies were performed using 10 nM [³H]MK801 and a 10⁻¹⁰ - 10⁻⁵ M range of MK801 concentrations. Saturation studies were carried out using a 0 - 40 nM range of [³H]MK801. For radioligand binding studies investigating the allosteric enhancement of [³H]MK801 by L-glutamate or glycine, the method for the preparation of cell homogenates was modified to remove endogenous glutamate and glycine. Thus, HEK 293 cell homogenates were prepared as described in section 2.2.24 and were then subjected to 3 additional cycles of homogenisation and centrifugation at 27,000 x g for 30 min at 4°C. Membrane pellets were resuspended to a protein concentration of 1.5 mg/ml and radioligand binding reactions carried out using 150 µg protein. [³H]MK801 radioligand binding (2 nM) was then determined in the presence of either 30 µM glycine and a 0 - 10 mM range of L-glutamate concentrations or 1 mM L-glutamate and a 0 - 100 µM range of glycine concentrations.

2.2.30.3 [³H]MDL 105,519 radioligand binding assays

[³H]MDL 105,519 radioligand binding assays were performed by incubating 10 - 15 µg protein in triplicate with [³H]MDL 105,519 and Tris-citrate buffer to a final volume of 200 µl. Non-specific binding was determined using 1 mM glycine. Incubations were carried out for 90 min at 4°C and terminated using the method of polyethylenimine filtration as previously described by Chazot *et al.* (1998). [³H]MDL 105,519 saturation assays were performed using a 0 - 10 nM range of [³H]MDL 105,519. Transfected cell homogenates for analysis by displacement of [³H]MDL 105,519 with glycine were prepared as described in section 2.2.24 except that an additional cycle of homogenisation and

centrifugation was carried out to remove excess glycine from the preparation. Displacement assays were performed using 3 nM [³H]MDL 105,519 and a 10⁻⁸ - 10⁻³ M range of glycine.

2.2.30.4 [³H]CGP 39653 radioligand binding assays

The method of HEK 293 cell homogenate preparation was modified for [³H]CGP 39653 binding assays according to Kendrick *et al.* (1996). The Tris-citrate buffer used in the method described in section 2.2.24 was replaced with Tris-HCl buffer (5 mM Tris-HCl, pH 7.7 at 4°C) which was filtered through a 0.2 µm filter unit to remove L-glutamate- or glycine-producing micro-organisms. Transfected HEK 293 cells were harvested and collected by the first cycle of centrifugation. The pelleted cells were processed by one cycle of homogenisation and centrifugation at 27,000 x g for 30 min at 4°C. The pellet was then resuspended by homogenisation in 50 ml Tris-HCl buffer supplemented with 10 mM EDTA (filtered and pre-warmed to 37°C) and incubated at 37°C for 10 min. Centrifugation and homogenisation was repeated for another 4 cycles at 4°C using the Tris-HCl buffer, and the pellet was resuspended to a final concentration of 1 mg protein/ml. Radioligand binding assays were performed by incubating 100 µg protein with [³H]CGP 39653 and 10 µM 7-CK in Tris-HCl buffer (5 mM Tris-HCl, pH 7.7 at 4°C) for 1 h in borosilicate glass tubes at 4°C. Non specific binding was determined using 100 µM L-glutamate. Reactions were terminated by polyethylenimine filtration over Whatman GF/B glass microfiber filters, pre-soaked in 0.3 % (v/v) polyethylenimine, followed by 3 x washes with 2 ml Tris-HCl buffer at 4°C. Competitive binding assays were performed by incubating 6 nM [³H]CGP 39653 with a 0 - 100 µM range of L-glutamate concentrations.

2.2.30.5 Analysis of radioligand binding data

Competition curves, saturation binding curves and dose-dependent enhancement curves were fitted and analysed using GraphPAD Prism software. Saturation radioligand binding data was analysed by either a one-site or a two-site binding hyperbola. To assess which binding model best fit the data, the F test was used. Dissociation constants (K_Ds) were determined by non-linear least squares regression analysis of saturation radioligand binding data. Competition curves were analysed by a sigmoidal dose response curve with a variable slope for the determination of Hill coefficients. To assess whether the data was best fit by a one-site or a two-site competition model the combined data from at least

n = 3 displacement experiments was analysed using the F test. IC₅₀ values were determined by non-linear regression analysis and apparent inhibition constants (K_Is) were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973):.

$$K_I = \frac{IC_{50}}{1 + \frac{L}{K_D}}$$

IC₅₀ = Inhibitory constant₅₀ for the non-radiolabelled ligand. The IC₅₀ is the concentration of non-radiolabelled ligand required to displace 50 % of a fixed concentration of competing radiolabelled ligand.

L = Concentration of radiolabelled ligand used in the competition assay.

K_D = Dissociation constant for the radiolabelled ligand determined in the absence of competing ligand.

K_I = Inhibition constant for the non-radiolabelled ligand. The K_I is equal to the concentration of non-radiolabelled ligand required to occupy 50 % of the binding sites in the absence of a competing ligand.

IC₅₀ values determined from homologous competitive binding analyses, *i.e.* the displacement of a [³H]MK801 by MK801, were converted to K_I values using a simplified version of the Cheng-Prusoff equation. Since the K_D and K_I for [³H]MK801 and MK801 respectively are equal, the equation simplifies to

$$K_I = IC_{50} - [L]$$

2.2.31 STATISTICAL SIGNIFICANCE ANALYSIS

The statistical significance of variations in parameters determined by quantitative immunoblotting and radioligand binding was tested using GraphPAD Prism Software. Raw data sets were compared by paired and unpaired student *t* tests as stated. The means of fold changes in immunoreactive intensities were tested for a significant deviation from a mean of unity.

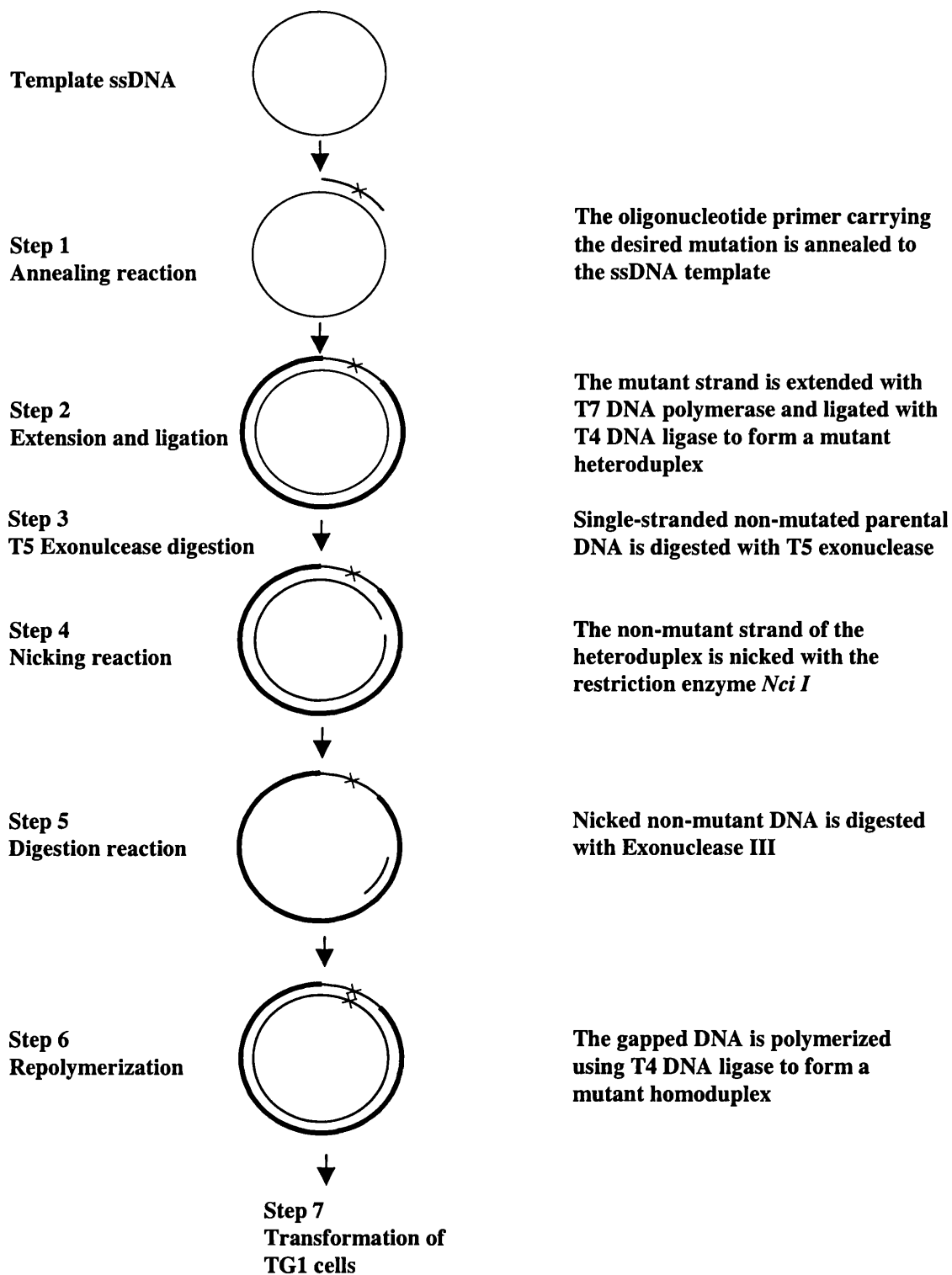


Figure 2.1 Schematic overview of the Sculptor™ *in vitro* mutagenesis system

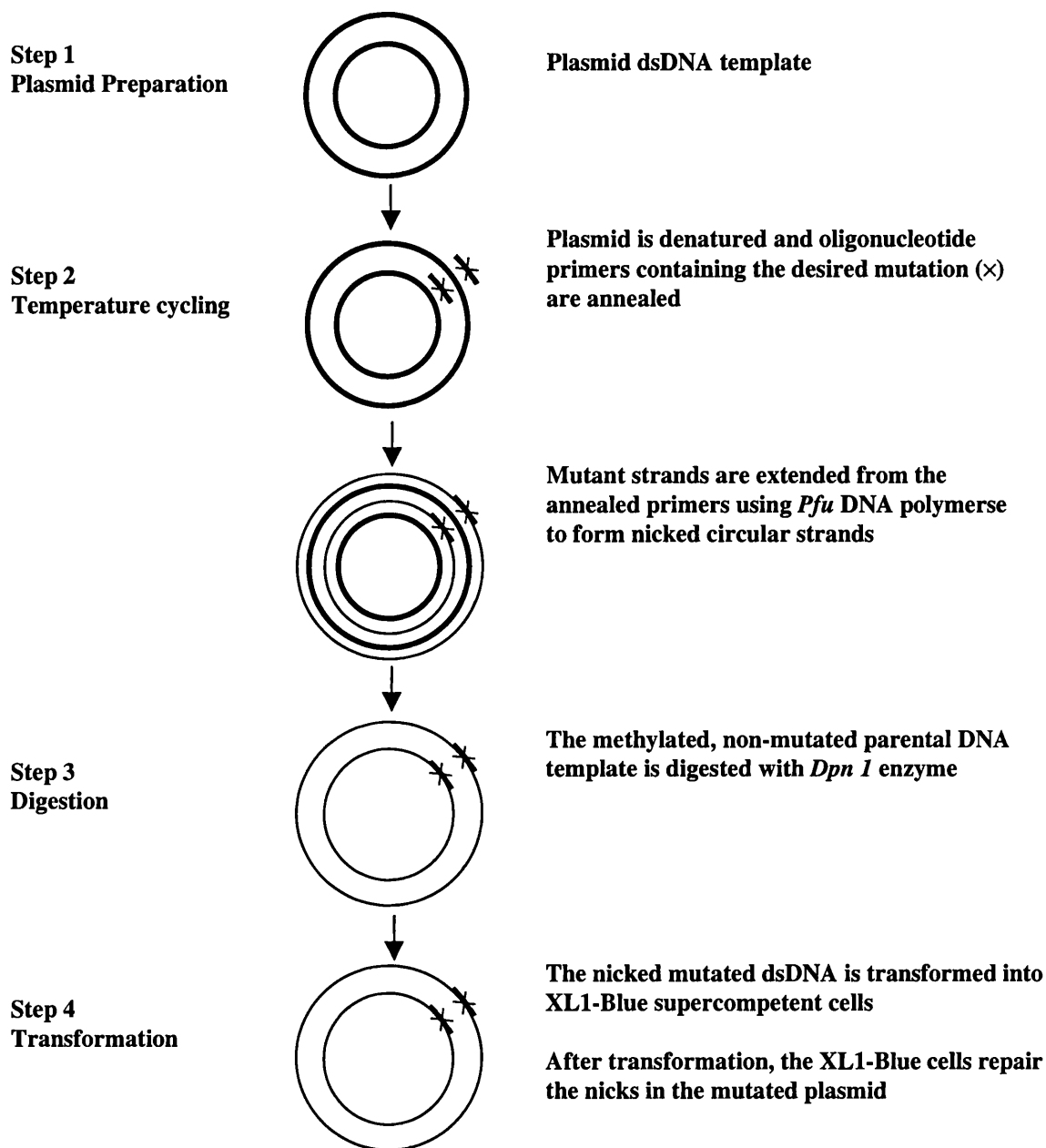


Figure 2.2 Schematic overview of the QuikChange™ method of site-directed mutagenesis

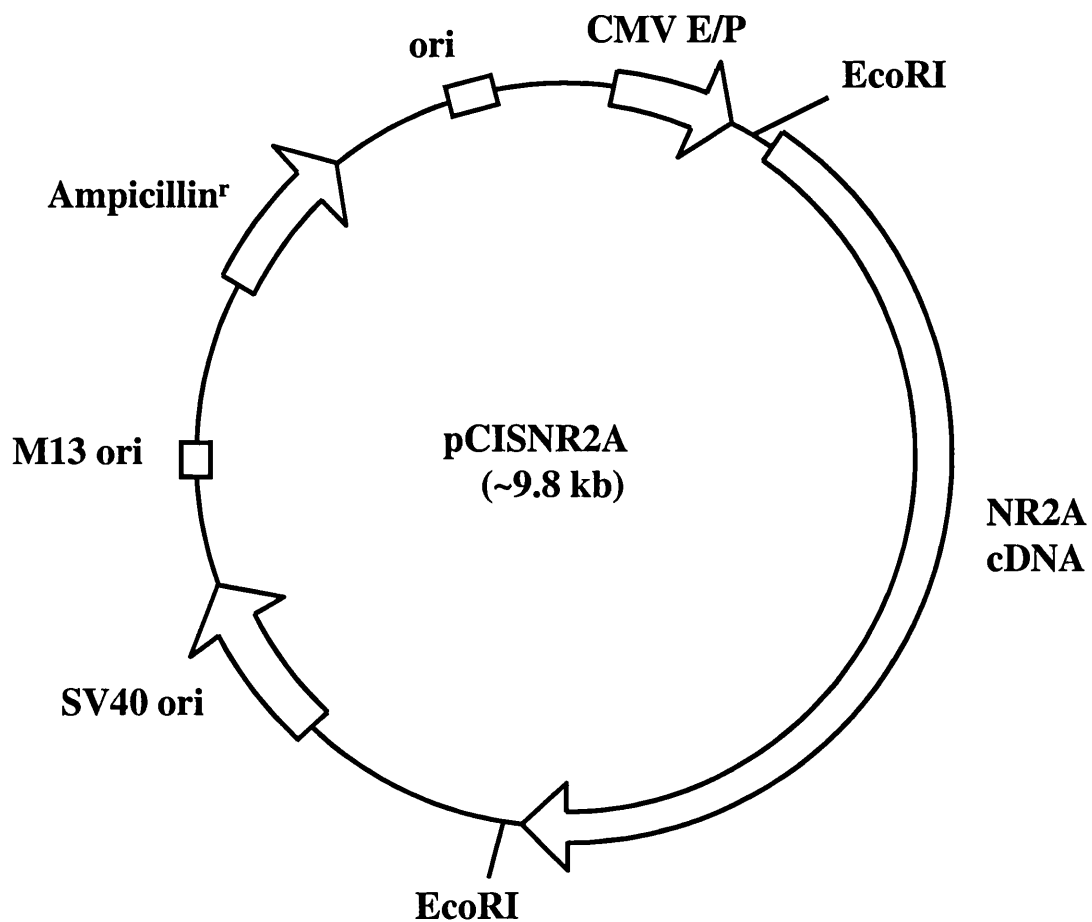


Figure 2.3 Restriction map of the vector pCISNR2A

The construct used for the generation of the pCISNR2A^{Trunc} mutant and for the transient expression of the mouse NR2A subunit in HEK 293 cells by transfection (Cik *et al.*, 1993). The *EcoRI* restriction sites shown were those used to screen recombinant clones for cDNA inserts corresponding to the size of NR2A. The open reading frame for the NR2A cDNA covers 4392 nucleotides. The size of pCISNR2A is shown as an approximation due to the presence of untranslated sequences 3' and 5' of the NR2A open reading frame.

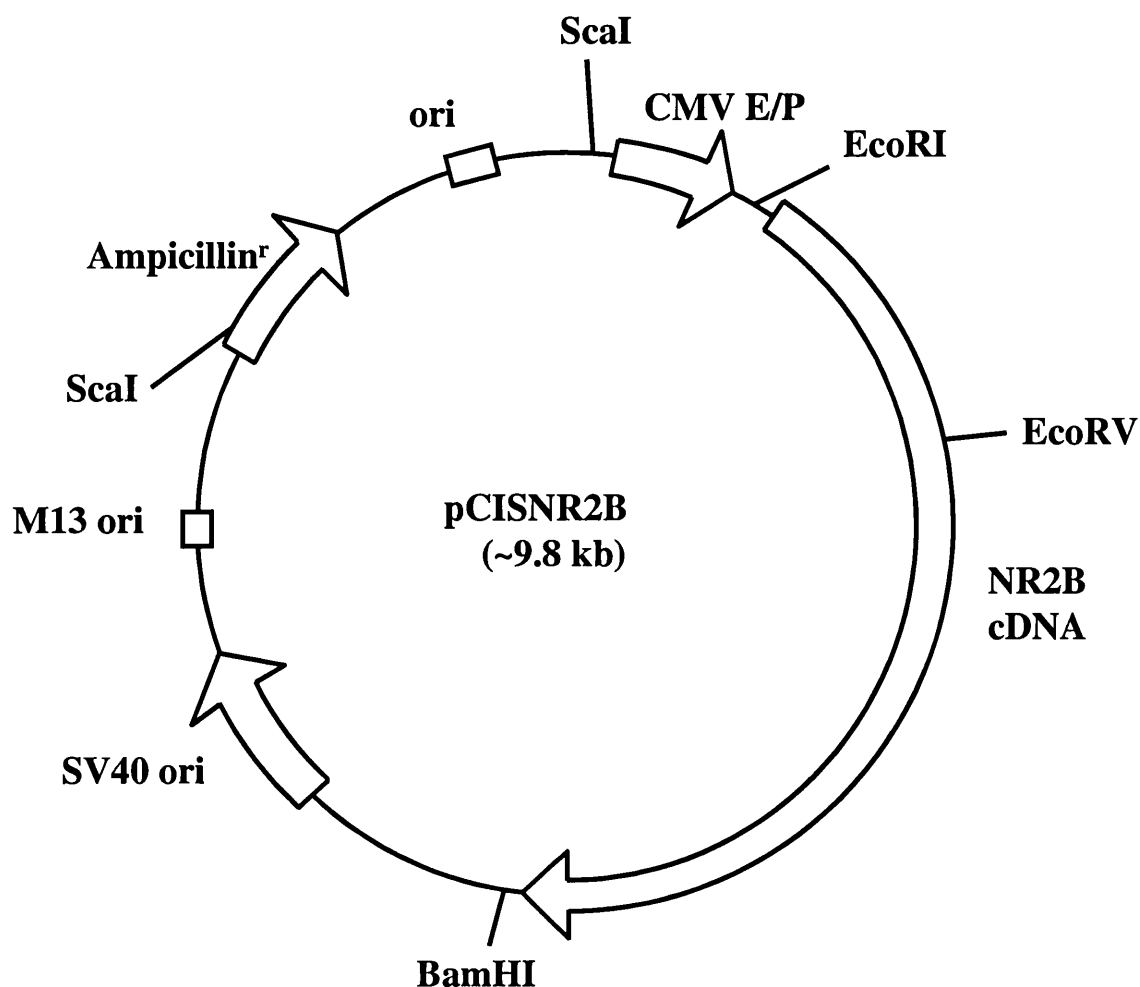


Figure 2.4 Restriction map of the vector pCISNR2B

The construct used for the generation of the NR2B^{Trunc} mutant and for the transient expression of the mouse NR2B subunit in HEK 293 cells. The NR2B cDNA was subcloned into the *EcoRI* and *BamHI* restriction sites of the pCIS multiple cloning sequence (Cik *et al.*, 1993). The locations of both the *EcoRV* restriction site in the NR2B sequence and the *ScaI* restriction site in the pCIS plasmid sequence are also shown. The open reading frame for the NR2B cDNA covers 4446 nucleotides. The size of pCISNR2B is shown as an approximation due to the presence of untranslated sequences 3' and 5' of the NR2B open reading frame.

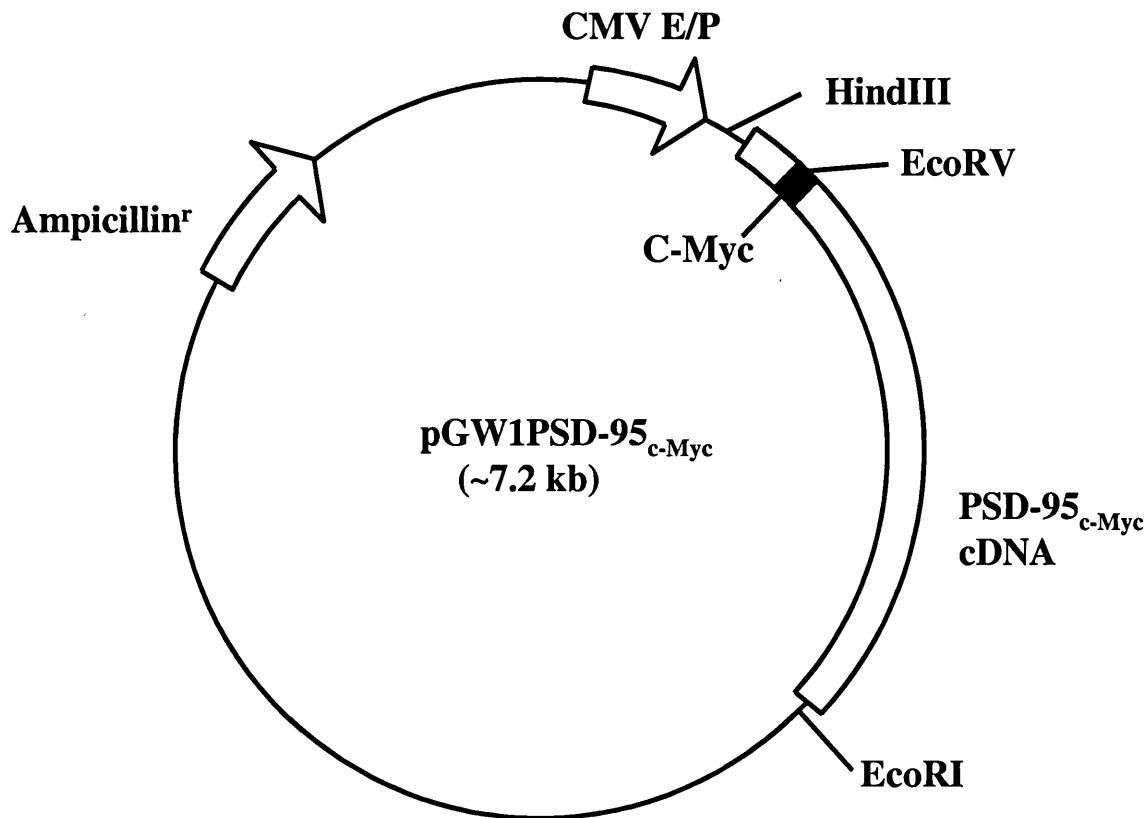


Figure 2.5 Restriction map of the vector pGW1PSD-95_{c-Myc}

The construct used for the generation of the PSD-95_{c-Myc}(C3S/C5S) mutant and for the transient expression of rat PSD-95_{c-Myc} in HEK 293 cells. The *EcoRI* and *HindIII* restriction sites were those used to screen recombinant clones for cDNA inserts corresponding to PSD-95_{c-Myc}. The PSD-95_{c-Myc} cDNA clone is 2298 nucleotides in length and the open reading frame begins at nucleotide 60, covering 2220 nucleotides. The pGWI plasmid is approximately 4.9 kb in length. The c-Myc tag cassette was inserted at nucleotide position 28 of the open reading frame and contains a unique *EcoRV* restriction site. The pGW1PSD-95 (non-tagged) vector is identical except for the presence of the c-Myc tag insert.

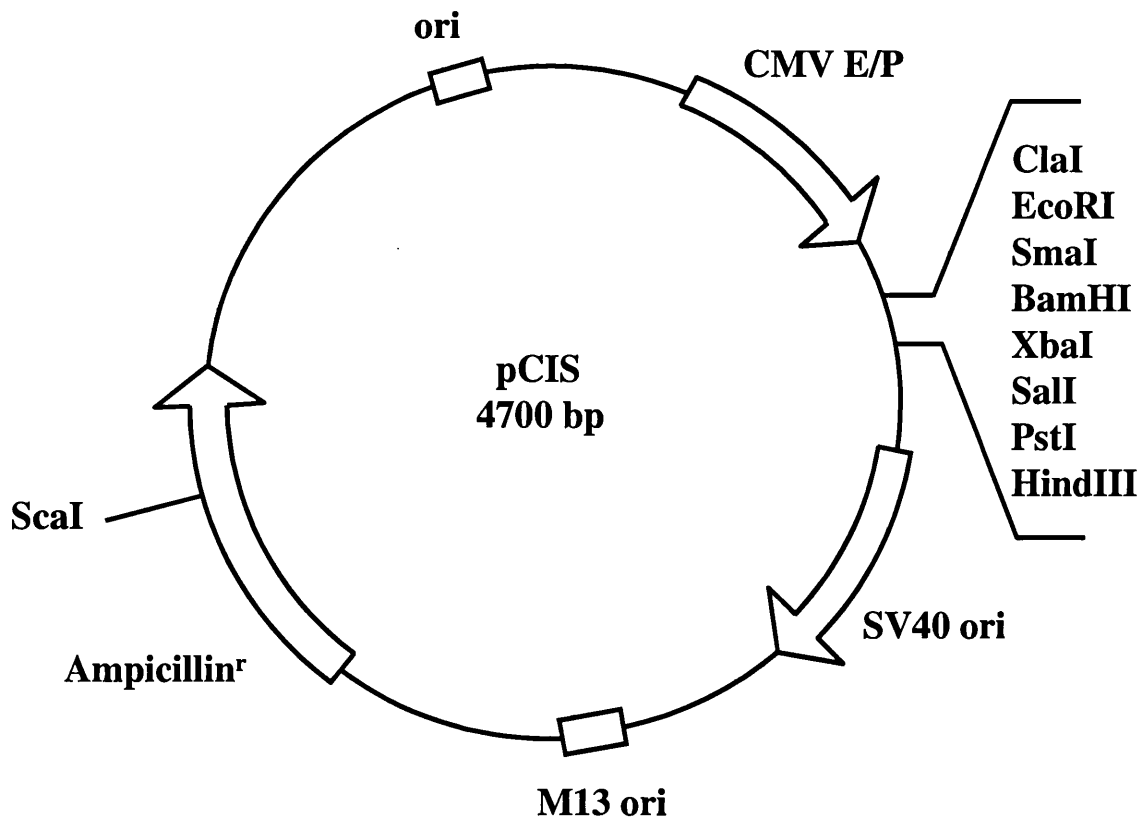


Figure 2.6 Restriction map of the plasmid pCIS

The plasmid pCIS is a mammalian expression vector containing the cytomegalovirus promoter/enhancer (CMV E/P) sequences. The vector has a multiple cloning site containing 8 unique restriction sites for subcloning. The bacterial origin of replication (ori) enables the plasmid to be efficiently replicated in *E. coli*. Transformed clones can be selected for ampicillin resistance.

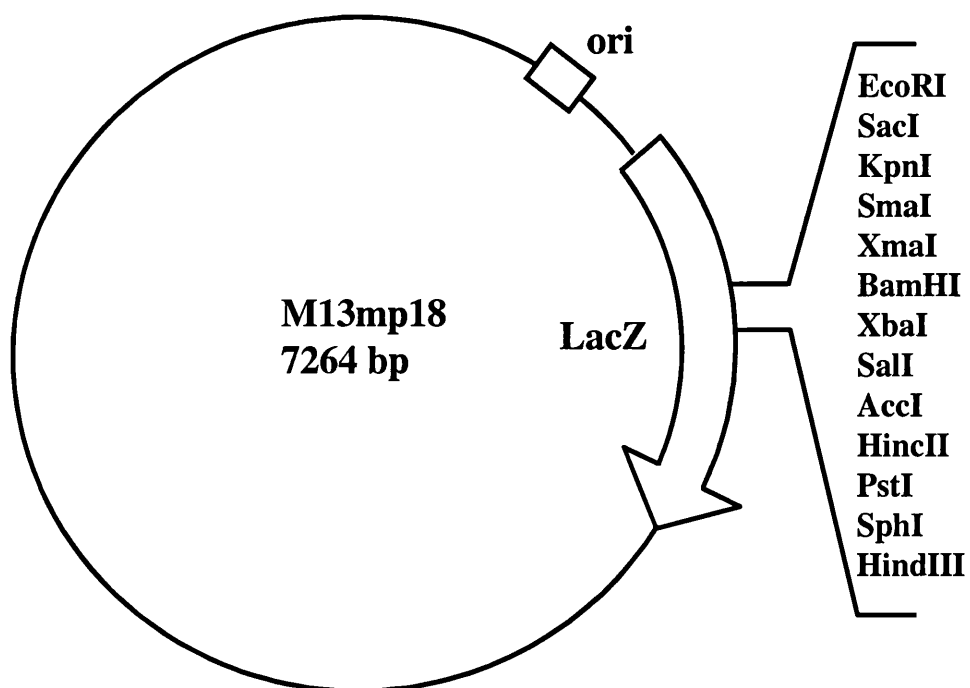


Figure 2.7 Restriction map of the plasmid m13mp18

The m13mp18 plasmid was used for the generation of ssDNA templates for mutagenesis reactions using the SculptorTM *in vitro* mutagenesis system. The plasmid M13mp18 encodes 11 phage genes. The vector has a multiple cloning site within the LacZ gene containing 13 unique restriction sites for subcloning. Transformed clones can be selected using the blue/white selection procedure.

Figure 2.8 Oligonucleotide primers for mutagenesis and sequencing reactions

Oligonucleotide primers were designed as described in 2.2.15.1 for the generation of the NR2B^{Trunc} subunit using the SculptorTM *in vitro* mutagenesis kit, and for the NR2A^{Trunc} and the PSD-95_{c-Myc}(C3S/C5S) mutants using the QuickchangeTM mutagenesis kit. The schematic shows the template DNA nucleotide sequence with the corresponding amino acid sequence, the oligonucleotide primer sequence and the resulting mutant DNA sequence. Nucleotides targeted for mutagenesis are underlined and highlighted in bold. M13mp18NR2B template DNA is ssDNA whereas the pCISNR2A and pGW1PSD-95_{c-Myc} DNA is dsDNA. For simplicity the dsDNA template sequences are shown in the 5' to 3' direction only. Oligonucleotide primers for the screening of possible mutant clones by automated nucleotide sequencing were designed as described in 2.2.15.1. The sequences of these primers, the direction of their sequence (*i.e.* plus or minus strand) and the positions of their complementary nucleotides in the appropriate cDNA and are shown in the figure.

Primer to generate NR2B^{Trunc}

Single point mutation at E1479

Template amino acids.....		L	S	S	I	E	S	D	V	
Template ssDNA(+)	5'	CTT	TCT	AGT	ATT	GAG	TCT	GAT	GTC	3'
Mutagenesis primer(-)	3'	GAA	AGA	TCA	TAA	ATC	AGA	CTA	CAG	5'
						↓				
Mutated DNA	5'	CTT	TCT	AGT	ATT	TAG	TCT	GAT	GTC	3'
Mutated amino acids		L	S	S	I	Stop				

Primers to generate NR2A^{Trunc}

Single point mutation at E1461

Complementary mutagenesis primers:

5'	CGT	GTG	TAC	AAG	AAA	ATG	CCT	AGT	ATT	TAA	TCT	GAT	GTC	TAA	GAT	C	3'
3'	GCA	CAC	ATG	TTC	TTT	TAC	GGA	TCA	TAA	ATT	AGA	CTA	CAG	ATT	CTA	G	5'

Template amino acids.....		S	P	S	I	E	S	D	V	
Template dsDNA	5'	ATG	CCT	AGT	ATT	GAA	TCT	GAT	GTC	3'
						↓				
Mutated dsDNA	5'	ATG	GCT	AGT	ATT	TAA	TCT	GAT	GTC	3'
Mutated amino acids		S	P	S	I	Stop				

Primers to generate PSD-95_{c-Myc}(C3S/C5S) mutant
Point mutations at C3 and C5

Complementary mutagenesis primers

```

5'      C CCC AAC ATG GAC AGT CTC AGT ATA GTG ACA ACC CGG CGC GCC GAG      3'
3'      G GGG TTG TAC CTG TCA GAG TCA TAT CAC TGT TGG GCC GCG CGG CTC      5'

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Template amino acids.....      M   D   C   L   C   I   V   T
Template DNA.....      5'   ATG GAC TGT CTC TGT ATA GTG ACA      3'
                               ↓           ↓
Mutated DNA.....      5'   ATG GAC AGT CTC AGT ATA GTG ACA      3'
Mutated amino acids.....      M   D   S   L   S   I   V   T

```

Sequencing Primers

```

NR2B.....      5'   GGG GCT GTG CCA GGT CGT TTC C      3'
Plus strand. Binds to nucleotides 4300-4322

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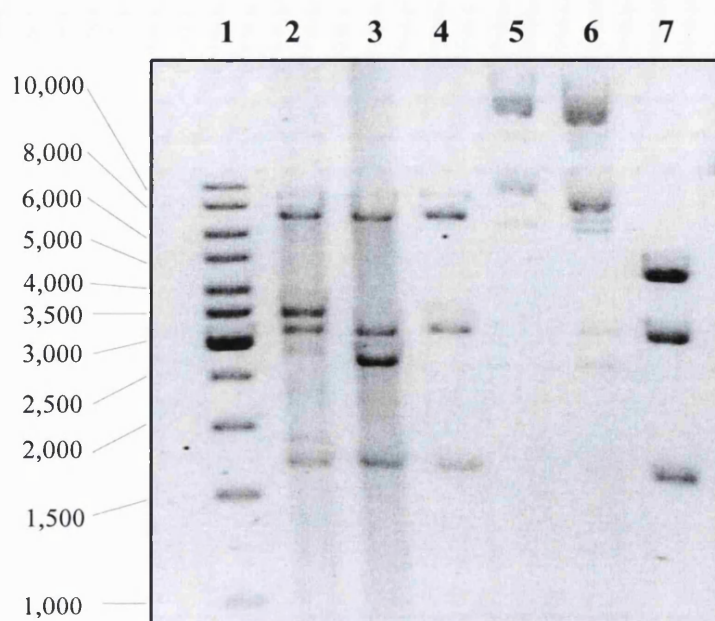
NR2A.....      5'   CGG GGC CAC AGT GAT GTG TAT ATT TC      3'
Plus strand. Binds to nucleotides 4249-4295

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PSD-95.....      5'   CCT GGG GCT TCT AGG GTG TCC GTG      3'
Minus strand. Binds to nucleotides 123-144

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Lane 1: Molecular weight standards

Lanes 2 - 6: Clones 1-5 digested with *EcoRI/BamHI/EcoRV*

Lane 7: pCISNR2B digested with *EcoRI/BamHI/EcoRV*

Figure 2.9 Restriction enzyme analysis of potential recombinant M13mp18NR2B clones

The entire coding region of the NR2B subunit clone was subcloned into *EcoRI/BamHI* restricted M13mp18 plasmid. Five possible recombinant clones (lanes 2 - 6) were selected using the blue/white selection procedure and screened by an *EcoRI/EcoRV/BamHI* restriction enzyme digest. Restriction enzyme digest products were analysed by flat-bed agarose gel electrophoresis as described in 2.2.8. The restriction digest of pCISNR2B with *EcoRI/EcoRV/BamHI* is shown in lane 7 to aid the comparison of NR2B restriction fragment. The positions of the molecular weight standards are shown on the left.

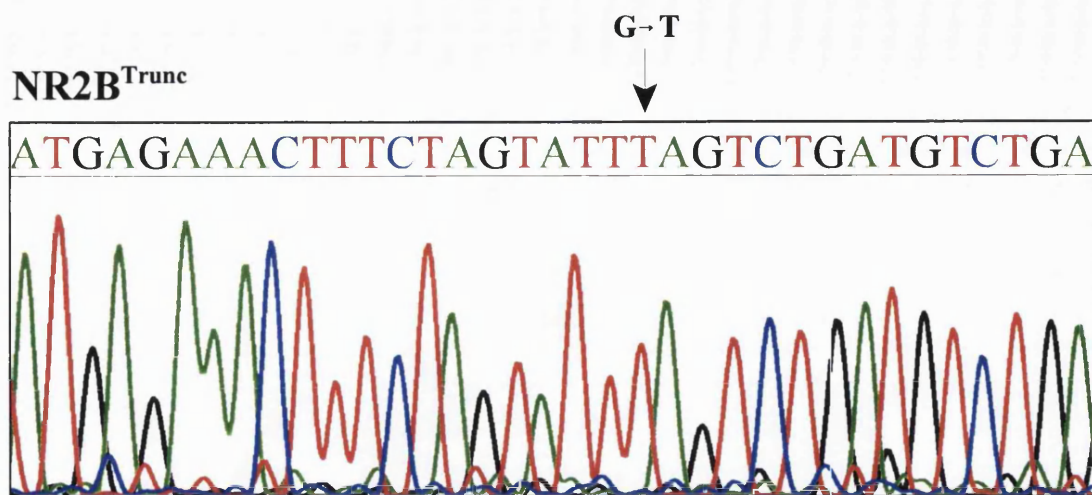
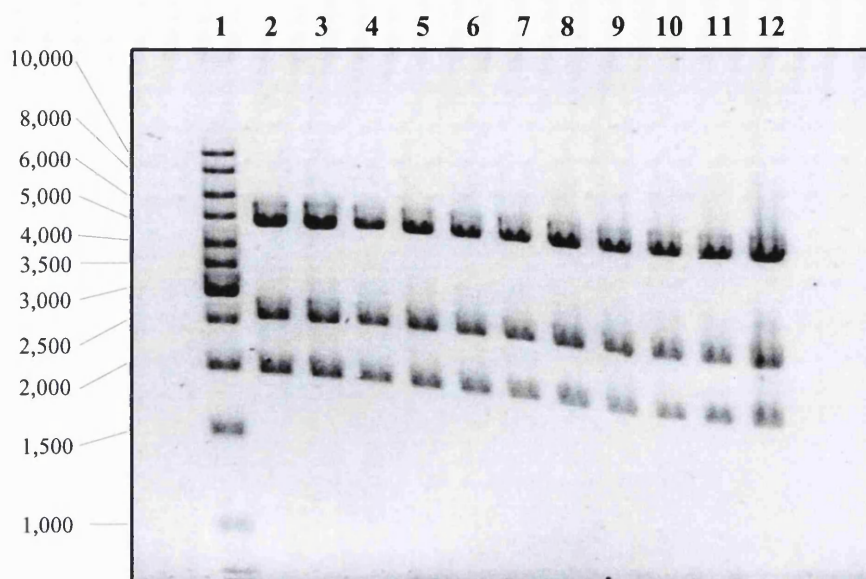


Figure 2.10 Automated nucleotide sequencing of M13mp18NR2B^{Trunc}

Mutagenesis reactions for the generation of NR2B^{Trunc} were carried out as described in section 2.16.3. Two potential mutant clones were screened by automated nucleotide sequencing using the oligonucleotide primers shown in Figure 2.8. One of the clones carried the desired G→T nucleotide substitution. The figure shows the sequence data for this positive clone, with the substituted nucleotide highlighted with an arrow.



- Lane 1: Molecular weight standards
 Lane 2-11: Clones 1-10 digested with *EcoRI/BamHI/ScaI*
 Lane 12: pCISNR2B digested with *EcoRI/BamHI/ScaI*

Figure 2.11 Restriction enzyme analysis of potential recombinant pCISNR2B^{Trunc} clones

The coding region for NR2B^{Trunc} was excised from M13mp18NR2B^{Trunc} and subcloned into pCIS digested with *EcoRI* and *BamHI*. Ten possible recombinant clones were selected and screened by restriction enzyme digestion with *EcoRI*, *BamHI* and *ScaI*. The positions of the DNA molecular weight standards are shown on the left.

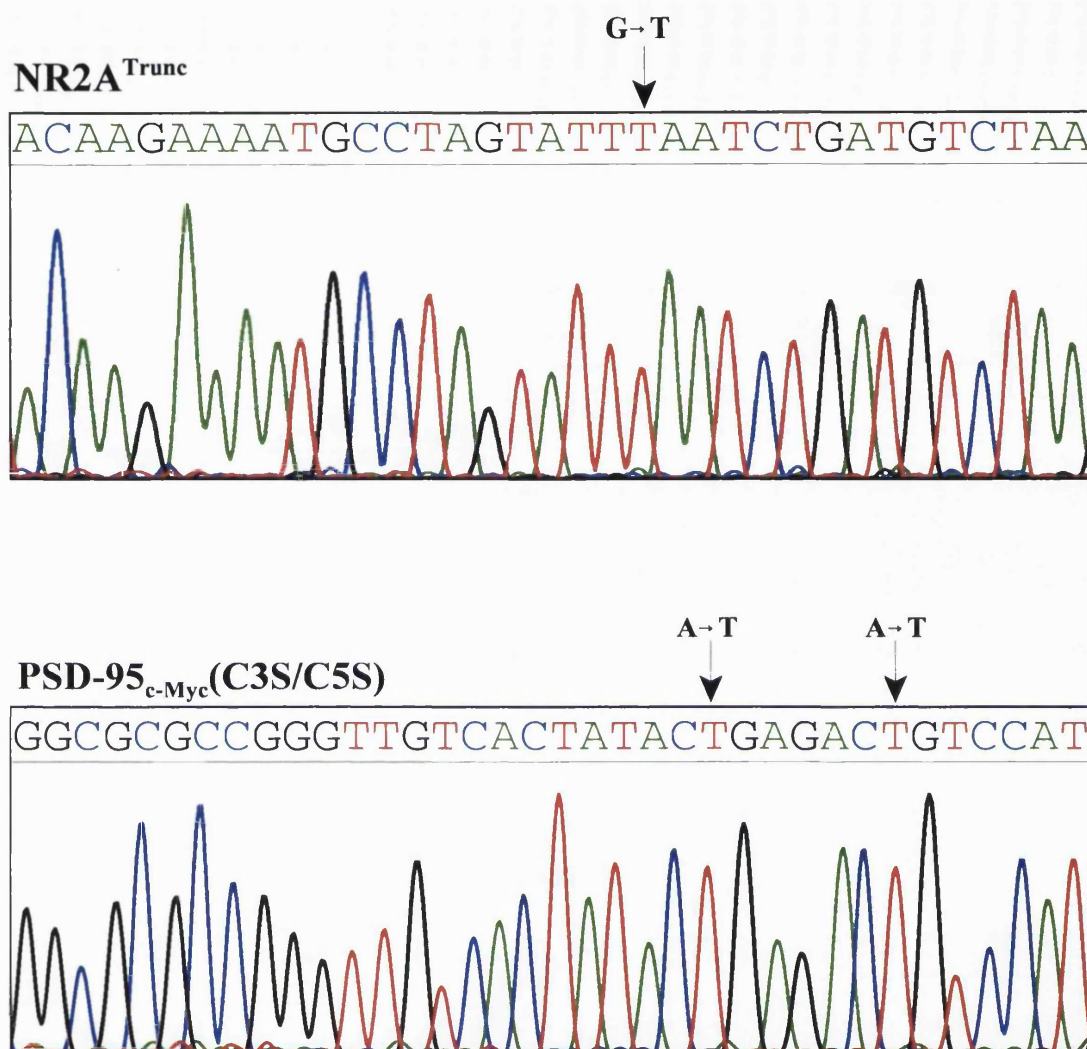


Figure 2.10 Automated nucleotide sequencing of pCISNR2A^{Trunc} and PSD-95_{c-Myc}(C3S/C5S) mutant clones

The mutagenesis reactions for the generation of NR2A^{Trunc} and PSD-95_{c-Myc}(C3S/C5S) were carried out as described in 2.16.3. Potential mutant clones were screened by automated nucleotide sequencing using the oligonucleotide primers shown in Figure 2.8. The figure shows the sequence data for a positive NR2A^{Trunc} and a PSD-95_{c-Myc}(C3S/C5S) clone, with the substituted nucleotides highlighted with an arrow. Note that the PSD-95_{c-Myc}(C3S/C5S) clone was sequenced in the reverse direction. The sequence shown therefore corresponds to the minus strand.

CHAPTER 3

Results

3.1 TRANSIENT EXPRESSION OF PSD-95 AND PSD-95_{c-Myc} IN HEK 293 CELLS AND CHARACTERISATION OF ANTI-PSD-95 ANTIBODIES

In order to investigate the effects of PSD-95 on the molecular pharmacological properties of NMDA receptors expressed in mammalian cells, a preliminary study was carried out to characterise the specificity of anti-PSD-95 (480-498) antibodies and to confirm the expression of both PSD-95 and PSD-95_{c-Myc} following transfection of HEK 293 cells.

Polyclonal anti-PSD-95 (480-498) antibodies were raised in two rabbits by immunization against the peptide, CSKRRVERRE, coupled to peptide carrier, thyroglobulin (carried out by Dr. P. L. Chazot). The rabbits were periodically bled and peptide-specific antibodies were purified from the immune serum by affinity chromatography. The specificity of the anti-PSD-95 (480-498) antibodies was determined by the immunoblot analysis of cell homogenates from both untransfected HEK 293 cells and HEK 293 cells transfected with pGW1PSD-95 as described in section 2.2.28. Antibodies purified from four consecutive bleeds from rabbit I and three bleeds from rabbit II were tested for immunoreactivity against PSD-95. Representative immunoblots are shown in Figure 3.1.1. The anti-PSD-95 (480-498) antibodies purified from bleeds II and IV from rabbit I and bleed 1 from rabbit II labelled an immunoreactive band from cells expressing PSD-95 with $M_r = 95 \pm 5$ kDa consistent with the reported molecular weight of PSD-95 (Kistner *et al.*, 1993). This band was absent from untransfected cells confirming the specificity of these antibodies for PSD-95. The absence of immunoreactivity in untransfected cells also showed that there was no endogenous expression of PSD-95 in HEK 293 cells. Two bands with $M_r = 120$ and $M_r = 140$ kDa labelled by anti-PSD-95 (480-498, bleed IV, rabbit I) antibodies were also detected in untransfected cells and were thus non-specific immunoreactive bands. All anti-PSD-95 (480-498) antibodies purified from other bleeds labelled only non-specific polypeptides with varying molecular weights.

The expression of c-Myc-tagged PSD-95 in HEK 293 cells was also verified by immunoblotting. HEK 293 cells transfected with pGW1PSD-95 and pGW1PSD-95_{c-Myc} were analysed by immunoblotting using the anti-c-Myc and anti-PSD-95 (480-498, bleed II, rabbit I) antibodies (Figure 3.1.2). The anti-c-Myc antibody detected an immunoreactive band with $M_r = 100 \pm 5$ kDa from HEK 293 cells transfected with pGW1PSD-95_{c-Myc}, that was absent from both untransfected cells and cells transfected with pGW1PSD-95. The anti-PSD-95 (480-498) antibody detected immunoreactive bands with $M_r = 100 \pm 5$ kDa

and $M_r = 95 \pm 5$ kDa from cells transfected with pGW1PSD-95_{c-Myc} and pGW1PSD-95 respectively, that were absent from untransfected cells. Thus, the expression of PSD-95_{c-Myc} in HEK 293 cells was confirmed. Furthermore, PSD-95_{c-Myc} was expressed to same level as the wild-type. However, a clear difference in the mobility between PSD-95 and PSD-95_{c-Myc}, representing an apparent shift in molecular weight of 5 kDa was observed as shown in Figure 3.1.2. The magnitude of this change in mobility does not correlate with the difference in the predicted molecular weight of the two polypeptides resulting from the insertion of the c-Myc tag, a 16 amino acid insert, and therefore may be an effect of the specific residues contained in the c-Myc tag on the electrophoretic properties of the PSD-95_{c-Myc} protein. In subsequent transfections of HEK 293 cells with PSD-95, the c-Myc tagged protein was expressed in preference to the wild-type PSD-95 due to the low specificity and availability of anti-PSD-95 antibodies.

3.2 THE TRANSIENT EXPRESSION OF SINGLE NMDA RECEPTOR SUBUNITS $\pm$ PSD-95_{c-Myc} IN HEK 293 CELLS

Initial experiments were performed to investigate the effect of the co-expression of PSD-95_{c-Myc} in HEK 293 cells on the expression level of single NR1-1a and NR2A subunits. It was clear from the analysis of resulting cell homogenates by quantitative immunoblotting that PSD-95_{c-Myc} altered the expression level of NR2A. Before further detailed analyses were carried out however, the reproducibility of the quantitative immunoblotting procedure was investigated to verify the accuracy of this method for the detection of changes in receptor subunit expression.

Verification of the reproducibility of quantitative immunoblotting

The relative quantification of NMDA receptor subunit expression in HEK 293 cells $\pm$ PSD-95_{c-Myc} by immunoblotting could be subject to a number of systematic errors. Significant variations in the efficiency of the transfection procedure would drastically reduce the accuracy of this quantification. In addition, the reproducibility of the immunoblotting procedure may be insufficient to resolve changes in receptor expression consistently. The contribution of these errors to the final quantification of NMDA receptor subunit expression was investigated to assess the suitability of this method.

The reproducibility of quantitative immunoblotting was determined by measuring the variability in OD measurements obtained by the quantitative analysis of three identical

samples of transfected HEK 293 cell homogenate. HEK 293 cells were transfected with pCISNR2A and analysed by quantitative immunoblotting using anti-NR2A (1,381-1,394) antibodies. A representative immunoblot is shown in Figure 3.2. The mean of the ODs $\pm$ SD obtained by the quantification of the NR2A immunoreactivities from these three samples was 628 ± 29 and the difference between the highest and the lowest value was 53. Therefore, the ODs did not vary by more than ± 10 % between identical samples.

The variability in the expression of NMDA receptor subunits between separately transfected samples of HEK 293 cells was also investigated. Replicate samples of HEK 293 cells were transfected in parallel with pCISNR1-1a. Cell homogenates were prepared and analysed by quantitative immunoblotting using anti-NR1 (911-920) antibodies (Figure 3.2). The mean OD of the NR1-1a immunoreactive bands was 854 ± 29 and the difference between the highest and the lowest value was 57. Therefore, the range of ODs obtained by the quantification of NR1-1a expressed in three separately transfected cell samples did not vary by more than ± 10 %.

The variability in both the transfection efficiency and in the quantification of immunoreactive intensities was minimal compared to the magnitude of the changes in expression of NMDA receptor subunits initially observed by the co-expression of PSD-95_{c-Myc}. However, as a precaution, the effect of the variability in quantitative immunoblotting was minimized by routinely analysing transfected samples in triplicate. Furthermore, to minimize the variation in transfection efficiency, three separately transfected flasks per receptor combination were routinely pooled before analysis by immunoblotting. The reproducibility of this method was judged to be acceptable for further comparisons of the relative level of NMDA receptor subunit expression in the presence and absence of PSD-95_{c-Myc}.

The effect of PSD-95_{c-Myc} on the expression level of single NR1-1a and NR2A subunits

HEK 293 cells were transfected with either NR1-1a or NR2A subunit clones in the presence and absence of pGW1PSD-95_{c-Myc}. Cell homogenates were analysed by quantitative immunoblotting to determine the expression level of NR1-1a and NR2A in the presence and absence of PSD-95_{c-Myc}. Representative immunoblots are shown in Figure 3.3. The anti-NR1 (911-920) antibody identified a single polypeptide band, $M_r = 117$ kDa, in cells transfected with NR1-1a, consistent with the molecular weight of N-glycosylated NR1-1a protein (Chazot *et al.*, 1992). The anti-NR2A (1,381-1,394) antibodies recognised a single polypeptide band, $M_r = 180$ kDa, in cells transfected with NR2A consistent with

the molecular weight of N-glycosylated NR2A subunit protein. Quantitative analysis of immunoreactive bands revealed that the NR2A subunit immunoreactivity was increased 3.4 ± 1.4 -fold ($n = 4$, $p < 0.05$, paired t test) in the presence of PSD-95_{c-Myc} compared to control transfections of NR2A in the absence of PSD-95_{c-Myc}. In contrast, the ratio of NR1-1a immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to intensities measured in the absence of PSD-95_{c-Myc} was 1.1 ± 0.1 ($n = 3$). The NR1-1a subunit was therefore not expressed at a significantly different level when expressed either in the presence or the absence of PSD-95_{c-Myc}.

3.3 THE TRANSIENT EXPRESSION OF NR1-1a/NR2A AND NR1-1a/NR2B RECEPTORS $\pm$ PSD-95 IN HEK 293 CELLS

The effect of PSD-95_{c-Myc} on the expression level of NR1 and NR2 subunits co-expressed in NR1/NR2 subunit combinations was investigated. Either NR1-1a/NR2A or NR1-1a/NR2B receptor clones were expressed in HEK 293 cells in the presence and absence of PSD-95_{c-Myc} and the resulting cell homogenates were analysed by quantitative immunoblotting using anti-NR1 (911-920), anti-NR2A (1,381-1394), anti-NR2B (1,435-1,445) and anti-c-Myc antibodies. Representative immunoblots are shown in Figure 3.4. The anti-NR2B antibody identified a single polypeptide, $M_r = 180$ kDa, in cells transfected with NR2B consistent with the molecular weight of N-glycosylated NR2B subunit protein (Chazot and Stephenson, 1997b). The additional band with $M_r \sim 100$ kDa labelled with this antibody is a non-specific protein because it is also detected in untransfected cells (Figure 3.4 B, lane 5). This band served as a useful internal control to verify that equal amounts of protein per gel lane were loaded. It was found that the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A and NR1-1a/NR2B subunit combinations resulted in a 2.9 ± 1.1 -fold ($n = 18$; $p < 0.001$, paired t test) and a 3.4 ± 1.4 -fold ($n = 8$; $p < 0.01$, paired t test) increase in the intensity of NR2A and NR2B subunit immunoreactivities respectively, with no concomitant change in the intensity of the NR1-1a subunit immunoreactivities. The ratio of the NR1-1a subunit immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to the intensities measured in the absence of PSD-95_{c-Myc} was 1.0 ± 0.2 ($n = 20$) for NR1-1a/NR2A receptors and 1.0 ± 0.2 ($n = 5$) for NR1-1a/NR2B receptors.

It is possible that this effect of PSD-95_{c-Myc} on the expression level of the NR2 subunit was in fact due to a non-specific effect of the control plasmid DNA that was routinely included in the transfection of NMDA receptor subunit clones in the absence of

PSD-95_{c-Myc}. As described in section 2.2.21, the transfection of NMDA receptor subunit clones was performed in parallel in the presence of either pGW1PSD-95_{c-Myc} or the control plasmid pSV- β -galactosidase. To determine whether β -galactosidase influenced the expression level of the NR2 subunit, an additional control was performed using pCIS vector in place of pSV- β -galactosidase. HEK 293 cells were transfected with NR1-1a/NR2A receptor clones in the presence of either pGW1PSD-95_{c-Myc} or pCIS vector. Cell homogenates were prepared and analysed by quantitative immunoblotting using anti-NR1 (911-920), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies. The intensity of the NR1-1a subunit immunoreactivity remained unchanged by the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A while the NR2A subunit immunoreactivity was increased by 2.3-fold (n = 1) in agreement with the enhanced expression obtained when pSV- β -galactosidase was used as the control plasmid DNA.

As described in 3.1, the c-Myc tagged PSD-95 was used routinely for the analysis of the effects of PSD-95 on NMDA receptor properties. To verify that PSD-95_{c-Myc} behaved as the wild-type PSD-95, NR1-1a/NR2A receptors were expressed in the presence and absence of PSD-95 and in the presence of PSD-95_{c-Myc}. The analysis of the resulting cell homogenates by quantitative immunoblotting, shown in Figure 3.5, revealed that the wild-type PSD-95 enhanced the NR2A subunit immunoreactivity to a similar extent compared to PSD-95_{c-Myc}. Neither the wild-type PSD-95 nor PSD-95_{c-Myc} altered the expression level of the NR1-1a subunit. Therefore, the previously observed effects of PSD-95_{c-Myc} on the expression of NR2A and NR2B was not due to a non-specific effect of the c-Myc tag.

3.4 THE EFFECT OF PSD-95_{c-Myc} ON NMDA RECEPTOR-MEDIATED CELL CYTOTOXICITY

The expression of NMDA receptors in HEK 293 cells results in cell death post-transfection. This effect is dependent upon the expression of functionally active NMDA receptors and therefore requires the co-expression of NR1 and NR2 subunits. A method for the quantification of the receptor-mediated cytotoxicity was initially developed to investigate both the ability of NMDA receptor antagonists to inhibit NMDA receptor function and to determine the effect of specific amino acid mutations on receptor function (Cik *et al.*, 1993; Cik *et al.*, 1994). The simplicity of this assay was therefore exploited to investigate the effect of PSD-95_{c-Myc} on the functional activity of NMDA receptors. HEK

293 cells were transfected with either NR1-1a/NR2A or NR1-1a/NR2B receptor clones in the presence and absence of PSD-95_{c-Myc} ± 1 mM ketamine. The resulting cell death was determined as described in section 2.2.23. The results are summarised in Table 3.1. The expression of PSD-95_{c-Myc} alone did not induce any significant cell death. The percentage cell death induced by NR1-1a/NR2A and NR1-1a/NR2B receptors in the absence of PSD-95_{c-Myc} was 22 % ± 3 (n = 3) and 25 % ± 3 (n = 2, ± range) respectively. This was in agreement with previously published data for the optimal expression of NMDA receptors in HEK 293 cells (Cik *et al.*, 1993). The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A and NR1-1a/NR2B receptors resulted in 20 % (n = 1) and 26 ± 2 % (n = 2, ± range) cell death respectively. Thus, any effect of PSD-95_{c-Myc} on the function of NR1-1a/NR2A and NR1-1a/NR2B receptors was not significant enough to alter the percentage NMDA receptor-mediated cell death post-transfection.

3.5 SINGLE POINT [³H]MK801 AND [³H]MDL 105,519 RADIOLIGAND BINDING TO NR1-1a/NR2A AND NR1-1a/NR2B RECEPTORS ± PSD-95_{c-Myc}

Preliminary radioligand binding studies were performed to investigate further the effect of PSD-95_{c-Myc} on the expression and pharmacological properties of NMDA receptor subunits in HEK 293 cells. Two NMDA receptor antagonists were utilised. The glycine site antagonist, [³H]MDL 105,519, was chosen to quantify the level of NR1-1a subunit expressed in the presence and absence of PSD-95_{c-Myc}, and the non-competitive channel site antagonist, [³H]MK801, which specifically binds to NMDA receptors co-assembled from NR1 and NR2 subunits, was chosen to investigate whether PSD-95_{c-Myc} altered the expression level of assembled NR1/NR2 NMDA receptors. Thus, NR1-1a/NR2A and NR1-1a/NR2B receptors were co-expressed in HEK 293 cells in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were assayed for [³H]MDL 105,519 (1 nM) and [³H]MK801 (25 nM) radioligand binding as described in section 2.2.30. The results are shown Figure 3.6. The specific [³H]MDL 105,519 binding to cells expressing NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc} was 1.5 ± 0.1 (n = 6) and 1.6 ± 0.1 (n = 5) pmol [³H]MDL 105,519 binding sites/mg protein respectively. The corresponding [³H]MK801 specific binding was 610 ± 160 (n = 6) and 410 ± 60 (n = 5) fmol [³H]MK801 binding sites/mg protein respectively. PSD-95_{c-Myc} therefore induced a significant (n = 6; *p* < 0.01, unpaired *t* test) increase of approximately 50 % in the number

of [^3H]MK801 binding sites to transfected cells expressing NR1-1a/NR2A. PSD-95_{c-Myc} did not however, induce a significant change in the number of [^3H]MDL 105,519 binding sites. These results suggest that PSD-95_{c-Myc} induced an increase in the level of co-assembled NR1-1a/NR2A receptor.

The specific [^3H]MDL 105,519 binding to cells expressing NR1-1a/NR2B in the presence and absence of PSD-95_{c-Myc} was 2.0 ± 0.1 (n = 3) and 2.0 ± 0.1 (n = 3) pmol [^3H]MDL 105,519 binding sites/mg protein respectively. However, [^3H]MK801 binding to NR1-1a/NR2B receptors yielded very low signal:noise ratios with non-specific binding accounting for up to 80 % of total [^3H]MK801 binding. This may be due to the reported three- to five-fold lower peak open probability for NR1-1a/NR2B compared to NR1-1a/NR2A (Chen *et al.*, 1999). Therefore, all future [^3H]MK801 binding experiments were carried out to cells transfected with NR1-1a/NR2A clones.

3.6 [^3H]MDL 105,519 AND [^3H]MK801 SATURATION RADIOLIGAND BINDING TO NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc}

In order to confirm the effects of PSD-95_{c-Myc} on the specific [^3H]MDL 105,519 and [^3H]MK801 binding to cells transfected with NR1-1a/NR2A receptors, full saturation analyses of [^3H]MDL 105,519 and [^3H]MK801 radioligand binding were carried out. [^3H]MDL 105,519 binding to NR1-1a/NR2A receptors expressed in HEK 293 cells in the presence and absence of PSD-95_{c-Myc} was performed as described in 2.2.30.3. Representative saturation curves and Rosenthal transformations are shown in Figure 3.7 and binding parameters are summarised in Table 3.2. [^3H]MDL 105,519 binding to NR1-1a/NR2A, both in the presence and absence of PSD-95_{c-Myc} was saturable and was not significantly best fit by a two-site binding model compared to a one-site binding model in all cases (*i.e.* $p > 0.05$, F test). Analysis of the curves yielded K_D values of 3.3 ± 0.1 nM (n = 2, $\pm$ range) and 3.5 ± 0.1 nM (n = 2, $\pm$ range) in the presence and absence of PSD-95_{c-Myc} respectively, with corresponding $B_{\max}$ values of 4.2 ± 1.2 and 4.2 ± 0.1 pmol of [^3H]MDL 105,519 binding sites/mg protein (both, mean of n = 2, $\pm$ range). Therefore, PSD-95_{c-Myc} did not significantly alter the number of [^3H]MDL 105,519 binding sites or the affinity of NR1-1a/NR2A receptors for [^3H]MDL 105,519.

[^3H]MK801 binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} was performed as described in 2.2.30.2 under standard assay conditions which were 10 μM glutamate and 30 μM glycine. Representative saturation curves and Rosenthal transformations are shown

in Figure 3.8 and the binding parameters are summarised in Table 3.2. [³H]MK801 binding to NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc} was best fit by a one-site model in all cases (*i.e.* $p > 0.05$, F test). Analysis of the binding curves by non-linear regression yielded K_D s of 37 ± 18 nM ($n = 5$) and 14 ± 4 nM ($n = 5$) in the presence and absence of PSD-95_{c-Myc} respectively. Therefore, the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors induced a significant 2.7 ± 1.0 -fold ($n = 5$; $p < 0.05$ unpaired t test) increase in the K_D for [³H]MK801. Corresponding B_{max} determinations were 920 ± 480 ($n = 3$) and 330 ± 130 ($n = 3$) pmol [³H]MK801 binding sites/mg protein in the presence and absence of PSD-95_{c-Myc} respectively. Thus, PSD-95_{c-Myc} induced a 2.8 ± 0.6 -fold ($n = 5$; $p < 0.05$ paired t test) increase in the B_{max} value for [³H]MK801 binding to NR1-1a/NR2A receptors.

Due to the decreased affinity of NR1-1a/NR2A receptors for [³H]MK801 in the presence of PSD-95_{c-Myc}, binding was not saturable up to concentrations of 40 nM. Affinity constants for MK801 binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} were therefore determined by displacement of [³H]MK801.

3.7 DETERMINATION OF THE AFFINITY OF NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} FOR MK801

The affinity of NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} expressed in HEK 293 cells and native NMDA receptors for MK801 was determined by the displacement of [³H]MK801 as described in 2.2.30.2. The results are shown in Figure 3.9 and the binding parameters are summarised in Table 3.2. The curves shown in Figure 3.9 for the displacement of [³H]MK801 from NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} were generated from the combined data of $n = 5$ separate transfections. Both curves were significantly best fit by a one-site binding model (+PSD-95_{c-Myc}, $p > 0.1$, F test; -PSD-95_{c-Myc}, $p > 0.5$, F test) with Hill coefficients of 1.08 ± 0.10 ($n = 5$) and 1.06 ± 0.13 ($n = 5$) for NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} respectively. The corresponding apparent inhibition constants (K_i s), calculated as described in 2.2.30.5, were 59 ± 19 nM ($n = 5$) and 12 ± 7 nM ($n = 5$) respectively. Therefore, PSD-95_{c-Myc} induced a 4.9 ± 1.6 -fold ($n = 5$; $p < 0.005$ unpaired t test) increase in the K_i value for MK801 binding to NR1-1a/NR2A receptors representing a significant decrease in the affinity of NR1-1a/NR2A receptors for MK801 in the presence of PSD-95_{c-Myc}. [³H]MK801 binding to rat forebrain membranes was also best fit by a one-site binding model with a Hill

coefficient of 0.92 ± 0.02 ($n = 3$) and a corresponding K_i value of 13 ± 8 nM ($n = 3$). The affinity of MK801 for rat forebrain membranes correlated well with the affinity of MK801 for NR1-1a/NR2A receptors, but was significantly ($n = 5$; $p < 0.05$, unpaired t test) higher than the observed affinity for NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc}.

3.8 ALLOSTERIC MODULATION OF [³H]MK801 BINDING TO NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} BY L-GLUTAMATE

[³H]MK801 is an open channel site, non-competitive, NMDA receptor antagonist. [³H]MK801 binding therefore requires the presence of L-glutamate and glycine in the radioligand binding assay. It has been shown that L-glutamate and glycine both modulate [³H]MK801 binding to rat brain cerebral cortex membranes by concentration-dependently increasing the affinity of NMDA receptors for [³H]MK801 (Ransom and Stec, 1988). This modulation of [³H]MK801 binding by L-glutamate and glycine is co-operative such that the sensitivity of [³H]MK801 binding to glutamate is enhanced by increasing concentrations of glycine and vice versa. The observed decrease in the affinity of NR1-1a/NR2A receptors for MK801 when co-expressed with PSD-95_{c-Myc} may therefore be due to a decrease in the sensitivity of [³H]MK801 binding to either L-glutamate or glycine. To test this hypothesis, the effect of PSD-95_{c-Myc} on the allosteric modulation of [³H]MK801 binding by L-glutamate to NR1-1a/NR2A receptors was investigated. However, it was first necessary to modify the [³H]MK801 radioligand binding assay to detect optimally the modulation of [³H]MK801 binding by L-glutamate.

Optimization of radioligand binding assays for the modulation of [³H]MK801 binding by L-glutamate

Since L-glutamate modulates the binding of [³H]MK801 to NMDA receptors by increasing the affinity of the receptor for [³H]MK801, it was important to select a suitable concentration of [³H]MK801 for the binding assay. A concentration of 2 nM was chosen since any effect of L-glutamate on the relative binding levels of [³H]MK801 due to changes in the affinity of [³H]MK801 would be greatest at low concentrations of [³H]MK801. However, it was critical that the specific binding of 2 nM [³H]MK801 to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} was high enough to accurately and reproducibly analyse the effect of PSD-95_{c-Myc} on the sensitivity of [³H]MK801 binding to L-glutamate. Therefore, the standard [³H]MK801 radioligand binding assay was modified to achieve optimal binding

of 2 nM [^{3} H]MK801. In addition, to observe significant enhancement of [^{3} H]MK801 binding by L-glutamate, it was necessary to remove endogenous L-glutamate from the transfected cell homogenates. Consequently, the method for the preparation of cell homogenates for the radioligand binding assay was modified to ensure the removal of endogenous L-glutamate.

NR1-1a/NR2A receptors were expressed in HEK 293 cells as described in 2.2.21. To remove endogenous L-glutamate transfected cell homogenates were washed by 5 cycles of homogenisation and centrifugation (as described in 2.2.30.2). Cell homogenates were then assayed for the binding of [^{3} H]MK801 (2 nM) in the presence and absence of 100 μ M L-glutamate. A concentration range of 50-200 μ g protein per 200 μ l of Tris-citrate buffer was assayed for [^{3} H]MK801 (2 nM) binding to establish a protein concentration resulting in high levels of specific binding at the low concentration of 2 nM while maintaining a linear relationship between the quantity of protein used and the specific bound radioligand measured. The results are shown in Figure 3.10 and Table 3.3.

The specific [^{3} H]MK801 binding measured in the presence 100 μ M L-glutamate was enhanced by approximately 3-fold at all protein concentrations compared to binding in the absence of added L-glutamate. The processing of cell homogenates by 5 x homogenisation and centrifugation was therefore sufficient to remove enough L-glutamate to study the allosteric modulation of [^{3} H]MK801 binding by L-glutamate. The level of non-specific binding was unchanged by the presence of 100 μ M L-glutamate.

The specific [^{3} H]MK801 binding to NR1-1a/NR2A receptors was directly proportional to the quantity of protein used in the radioligand binding assay. Therefore, all the protein concentrations assayed were acceptable. The percentage contribution of non-specific binding to the total [^{3} H]MK801 binding was however, significantly less when higher concentrations of protein were used in the binding assay. For example, in the presence of 200 μ g protein and 100 μ M L-glutamate, non-specific binding accounted for 24 ± 2 % of the total binding compared to 48 ± 5 % in the presence of 50 μ g protein and 100 μ M L-glutamate. A high percentage of non-specific binding would introduce unnecessary errors into the final determination of the specific [^{3} H]MK801 binding. Consequently, radioligand binding assays investigating the allosteric effects of L-glutamate on the binding of 2 nM [^{3} H]MK801 were routinely performed on 150 μ g protein samples.

The effect of PSD-95_{c-Myc} on the allosteric modulation of [³H]MK801 binding by L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A receptor clones in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were prepared as described in 2.2.30.2 to remove endogenous L-glutamate and to optimize the binding of 2 nM [³H]MK801. [³H]MK801 binding (2 nM) to transfected HEK 293 cell homogenates and to rat forebrain membranes was measured in the presence of a 0 - 10 mM range of L-glutamate concentrations. Glycine was included in all assay tubes at a saturating concentration of 30 μ M. The results are shown in Figure 3.11 and the binding parameters are summarised in Table 3.4. L-Glutamate enhanced [³H]MK801 binding to NR1-1a/NR2A receptors in a dose-dependent manner in both the presence and the absence of PSD-95_{c-Myc}. The concentrations of L-glutamate required to elicit 50 % maximum enhancement, *i.e.* the EC₅₀ values, were $7.8 \pm 1.7 \mu\text{M}$ (n = 3) and $1.8 \pm 0.4 \mu\text{M}$ (n = 5) in the presence and absence of PSD-95_{c-Myc} respectively. The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A therefore resulted in a significant (n = 3; $p < 0.001$, unpaired *t* test) increase in the EC₅₀ values representing an approximate 5-fold decrease in the sensitivity of [³H]MK801 binding to L-glutamate. The enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} elicited by saturating concentrations of L-glutamate were $240 \pm 70 \%$ (n = 3) and $220 \pm 60 \%$ (n = 5) respectively. PSD-95_{c-Myc} did not therefore significantly alter the maximum percentage enhancement of [³H]MK801 binding by L-glutamate.

L-glutamate enhanced [³H]MK801 binding to rat forebrain membranes dose-dependently with an EC₅₀ value of $0.46 \pm 0.04 \mu\text{M}$ (n = 3) and to a maximum enhancement of $130 \pm 30 \%$ (n = 3). Therefore, native NMDA receptors exhibited a significantly greater sensitivity of [³H]MK801 binding to L-glutamate compared to NR1-1a/NR2A receptors both in the presence (n = 3, $p < 0.005$, unpaired *t* test) and the absence (n = 3; $p < 0.005$, unpaired *t* test) of PSD-95_{c-Myc}.

It is apparent that PSD-95 also altered the Hill slope for the enhancement of [³H]MK801 binding by L-glutamate to NR1-1a/NR2A receptors (see Table 3.4). In the presence of PSD-95, the Hill coefficient was not significantly different from unity whereas in the absence of PSD-95 the Hill coefficient was 1.45 ± 0.25 (n = 5). An intermediate value of 1.22 ± 0.06 (n = 3) was obtained for the enhancement of [³H]MK801 binding to rat forebrain membranes.

An important control for the effect of PSD-95_{c-Myc} on the L-glutamate sensitivity

of [^3H]MK801 binding was the substitution of PSD-95_{c-Myc} with the wild-type PSD-95. Full dose-response curves for the L-glutamate enhancement of [^3H]MK801 binding were not performed for this analysis or indeed for future analyses, due to the impracticability of preparing such large quantities of transfected cell homogenate. Owing to the reproducibility and accuracy of the modified [^3H]MK801 binding assay, the modulation of [^3H]MK801 binding by L-glutamate was instead analysed by determining the specific [^3H]MK801 binding (2 nM) in the presence of 0, 10 μM and 1 mM L-glutamate. The subsequent specific binding measured at 1 mM glutamate and 10 μM was adjusted to a percentage enhancement of the binding in the absence of added L-glutamate. The ratio of the percentage enhancement at 1 mM L-glutamate to the percentage enhancement at 10 μM L-glutamate was then calculated. For NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}, 10 μM and 1 mM L-glutamate were both saturating, resulting in a ratio of 1. However, for NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc}, the percentage enhancement of [^3H]MK801 binding at 10 μM L-glutamate was only just above 60 %, whereas at 1 mM L-glutamate, [^3H]MK801 binding was maximally enhanced, resulting in a ratio of 1.4 - 1.8. This assay significantly reduced the quantities of cell homogenate required to perform the analysis and permitted the simultaneous screening of multiple combinations of receptor subtypes for their sensitivity to L-glutamate. Hence, the enhancement ratios for the modulation of [^3H]MK801 binding to NR1-1a/NR2A receptors in the presence of PSD-95 and PSD-95_{c-Myc} were 1.44 (n = 1) and 1.42 (n = 1) respectively, whereas to NR1-1a/NR2A receptors in the absence of PSD-95 the enhancement ratio was 0.99 (n = 1). Therefore, with respect to its ability to decrease the sensitivity of [^3H]MK801 binding to L-glutamate the c-Myc tagged PSD-95 behaved as the wild-type. In the same experiment it was observed that wild-type PSD-95 and PSD-95_{c-Myc} increased the single point [^3H]MK801 binding (2 nM) by 130 % and 160 % respectively compared to single point binding to NR1-1a/NR2A receptors alone. Therefore, with respect to the ability of PSD-95 to increase the number assembled receptors, as determined by single point [^3H]MK801 binding, the wild-type PSD-95 behaved identically to the PSD-95_{c-Myc}.

3.9 ALLOSTERIC MODULATION OF [^3H]MK801 BINDING TO NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} BY GLYCINE

The observed decrease in affinity of NR1-1a/NR2A receptors for MK801 in the presence of PSD-95_{c-Myc} may also be due to a decrease in the sensitivity of the receptor for

glycine. The allosteric modulation of [³H]MK801 binding by glycine was therefore investigated.

Well-washed HEK 293 cell homogenates expressing NR1-1a/NR2A receptors ± PSD-95_{c-Myc} were prepared as described in 2.2.30.2 and [³H]MK801 radioligand binding (2 nM) was assayed in the presence of a range of glycine concentrations (0-100 μM). L-Glutamate was included in all assay tubes at a final concentration of 1 mM which was saturating for [³H]MK801 binding to NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc}. It is notable that in the absence of added L-glutamate glycine did not enhance [³H]MK801 binding above non-specific levels of [³H]MK801 binding. Representative enhancement curves are shown in Figure 3.12 and the binding parameters are summarised in Table 3.4. Glycine enhanced the binding of [³H]MK801 to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} with EC₅₀ values of 270 ± 50 nM (n = 5) and 67 ± 30 nM (n = 4) respectively. The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors therefore significantly (n = 5; *p* < 0.001, unpaired *t* test) increased the EC₅₀ value representing a 6.1 ± 4.6-fold decrease in the sensitivity of [³H]MK801 binding to glycine. For both curves the Hill coefficients were greater than unity, *i.e.* 1.33 ± 0.23 (n = 5) and 1.48 ± 0.17 (n = 5) in the presence and absence of PSD-95_{c-Myc} respectively. The corresponding maximum percentages of enhancement elicited by saturating concentrations of glycine were 140 ± 80 % (n = 5) and 68 ± 63 % (n = 5) in the presence and absence of PSD-95_{c-Myc} respectively. Therefore, it was apparent that the maximum percentage enhancement elicited by glycine was lower in the absence of PSD-95_{c-Myc}. Indeed, in some cases very little percentage enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors (minus PSD-95_{c-Myc}) was actually observed. Importantly, the low levels of enhancement detected on these occasions were due to high levels of [³H]MK801 binding in the absence of added glycine rather than a decrease in the maximum specific [³H]MK801 binding determined at saturating concentrations of glycine. Therefore, the decrease in the percentage enhancement detected in the absence of PSD-95_{c-Myc} may be due to a greater sensitivity of these receptors to residual glycine compared to receptors in the presence of PSD-95_{c-Myc}. If the residual concentration of glycine was close to saturating concentrations for [³H]MK801 binding to NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}, then further addition of glycine would not considerably enhance [³H]MK801 binding. Consequently a decreased maximum percentage enhancement of [³H]MK801 binding to receptors in the absence of PSD-95_{c-Myc} would be observed. In an attempt to reduce contamination by endogenous glycine, the transfected cell

homogenates were processed by a further two cycles of homogenisation and centrifugation. However, this did not increase the maximum percentage enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}.

Enhancement of [³H]MK801 binding to rat forebrain membranes by glycine was not detected. This was also probably due to the effect of residual glycine in the membrane preparation. To establish whether this reduced enhancement of [³H]MK801 binding by glycine to both NR1-1a/NR2A receptors and native NMDA receptors, was indeed due to a greater sensitivity of these receptors to residual glycine, the enhancement of [³H]MK801 binding by glycine was investigated in the presence of a fixed concentration of the glycine site antagonist, 7-CK.

3.10 THE EFFECT OF 10 μ M 7-CHLOROKYNURENATE ON THE ALLOSTERIC MODULATION OF [³H]MK801 BINDING TO NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} BY GLYCINE

In rat forebrain preparations, glycine dose-dependently enhances the binding of [³H]MK801 (Ransom and Stec, 1988). These concentration-effect curves were shifted to the right by the glycine site antagonist, 7-CK, which competes with glycine for its binding to the NMDA receptor (Peeters and Vanderheyden, 1992). It was therefore predicted that the enhancement curves for the modulation of [³H]MK801 binding by glycine to NR1-1a/NR2A receptors (described in 3.9) would be shifted to the right by including a fixed concentration of 7-CK. Under these conditions, the contribution of residual glycine in the transfected cell homogenate to the enhancement of [³H]MK801 binding would be drastically reduced. Therefore, a concentration of 7-CK was selected that would shift the EC₅₀ value for the glycine enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors + PSD-95_{c-Myc} from 270 ± 50 nM (determined in 3.9) to approximately 10 μ M.

NR1-1a/NR2A receptors were expressed in HEK 293 cells in the presence of PSD-95_{c-Myc}. Cell homogenates were incubated with [³H]MK801 (2 nM), 10 μ M glycine, 1 mM L-glutamate and a range of 7-CK (0 - 1 mM). The result is shown in Figure 3.13. The concentration of 7-CK that displaced [³H]MK801 binding to 50 % was approximately 10 μ M.

The enhancement of [³H]MK801 binding by glycine to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} and to rat forebrain membranes was then analysed in the presence of 10 μ M 7-CK and 1 mM L-glutamate. Representative enhancement curves are shown in Figure 3.14

and binding parameters are summarised in Table 3.4. The maximum percentage enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} and to rat forebrain membranes elicited by saturating concentrations of glycine were $660 \pm 210 \%$ ($n = 3$), $720 \pm 70 \%$ ($n = 3$) and $510 \pm 250 \%$ ($n = 3$) respectively. Therefore, PSD-95_{c-Myc} did not significantly alter the maximum percentage enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors when binding was performed in the presence of 10 μ M 7-CK. The previously observed difference in the maximum percentage enhancement in section 3.9 was therefore due to the effects of residual glycine in the cell homogenate. EC₅₀ values for the enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} and to rat forebrain membranes were $3.1 \pm 0.7 \mu$ M ($n = 3$), $7.3 \pm 1.5 \mu$ M ($n = 3$) and $0.67 \pm 0.06 \mu$ M ($n = 3$) respectively. Therefore, PSD-95_{c-Myc} significantly ($n = 3$; $p < 0.05$, unpaired t test) decreased the sensitivity of NR1-1a/NR2A receptors to glycine in the presence of 10 μ M 7-CK. Furthermore, [³H]MK801 binding to native receptors was significantly more sensitive to glycine compared to NR1-1a/NR2A ($n = 3$; $p < 0.01$, unpaired t test) and NR1-1a/NR2A/PSD_{c-Myc} ($n = 3$; $p < 0.01$, unpaired t test).

The Hill coefficients for the enhancement of [³H]MK801 by glycine were also dependent upon the receptor preparation. For [³H]MK801 binding to NR1-1a/NR2A receptors in the presence of PSD-95, the Hill coefficient of 1.21 ± 0.02 ($n = 3$) was the closest to unity, whereas in the absence of PSD-95 with Hill coefficient was increased to 1.50 ± 0.17 ($n = 3$). The value obtained for rat forebrain membranes was 1.59 ± 0.08 ($n = 3$).

3.11 THE EFFECT OF GLYCINE ON THE ALLOSTERIC MODULATION OF [³H]MK801 BINDING TO NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} BY L-GLUTAMATE

The allosteric modulation of [³H]MK801 binding by both L-glutamate and glycine revealed that PSD-95_{c-Myc} induced a decrease in the sensitivity of the NR1-1a/NR2A receptor for both L-glutamate and glycine. The enhancement of [³H]MK801 binding by L-glutamate (as in Figure 3.11) was performed in the presence of 30 μ M glycine, which was assumed to be saturating for [³H]MK801 binding at all L-glutamate concentrations. Even though 30 μ M glycine was indeed saturating for [³H]MK801 binding in the presence of 1 mM L-glutamate (shown in Figure 3.12), it may be limiting at lower concentrations of

L-glutamate since glycine and L-glutamate co-operatively enhance [^{3} H]MK801 binding (Ransom and Stec, 1988). Consequently, the rightward shift in the curve for the L-glutamate enhancement of [^{3} H]MK801 binding induced by PSD-95_{c-Myc} may actually be due to the decrease in the sensitivity of NR1-1a/NR2A receptors to glycine in the presence of PSD-95_{c-Myc}. Therefore, the effect of PSD-95_{c-Myc} on the sensitivity of NR1-1a/NR2A receptors to L-glutamate was re-determined in the presence of 1 mM glycine.

The percentage enhancement ratios for [^{3} H]MK801 binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} by L-glutamate in the presence of either 30 μ M or 1 mM glycine were determined as described in section 3.8. For NR1-1a/NR2A - PSD-95_{c-Myc}, they were 1.02 (n = 1) and 0.96 (n = 1) in the presence of 30 μ M and 1 mM glycine respectively, and for NR1-1a/NR2A + PSD-95_{c-Myc} they were 1.47 (n = 1) and 1.42 (n = 1) respectively. The PSD-95_{c-Myc}-mediated decrease in the sensitivity of NR1-1a/NR2A receptors to L-glutamate as determined by the enhancement of [^{3} H]MK801 binding was not therefore due to a change in sensitivity to glycine.

3.12 THE EFFECT OF L-GLUTAMATE ON THE AFFINITY OF NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} FOR MK801

Preliminary experiments described in section 3.7 revealed that the co-expression of PSD-95_{c-Myc} induced a decrease in the affinity of NR1-1a/NR2A receptors for MK801 in the presence of 10 μ M L-glutamate and 30 μ M glycine. These concentrations of L-glutamate and glycine were known to be saturating for the binding of [^{3} H]MK801 to rat cerebral cortex membranes, and were thus the conditions of choice for [^{3} H]MK801 binding to recombinant NR1-1a/NR2A receptors (Chazot *et al.*, 1992). However, it is clear from the L-glutamate enhancement curves shown in Figure 3.11 that although 10 μ M L-glutamate was saturating for [^{3} H]MK801 binding to NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}, this concentration of L-glutamate elicited only just over 50 % of the maximum enhancement of [^{3} H]MK801 binding to NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc}. Therefore, the affinity of MK801 for NR1-1a/NR2A receptors + PSD-95_{c-Myc} determined in section 3.7 was measured under non-saturating concentrations of L-glutamate. With respect to glycine, the enhancement curves shown in Figure 3.12 revealed that 30 μ M glycine was saturating for [^{3} H]MK801 binding to NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc}. To investigate whether the observed decrease in affinity of NR1-1a/NR2A receptors for MK801 in the presence of

PSD-95_{c-Myc} was indeed due to a decrease in the sensitivity of MK801 binding to L-glutamate, the affinity of NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} for MK801 was determined by competition assay in the presence of either 10 μ M or 1 mM L-glutamate. Representative curves are shown in Figure 3.15 with K_s s summarised in Figure 3.16. The K_s s for the displacement of [³H]MK801 by MK801 from NR1-1a/NR2A receptors were 59 ± 19 (n = 5) and 13 ± 9 nM (n = 3) in the presence of PSD-95_{c-Myc} at 10 μ M L-glutamate and 1 mM L-glutamate respectively, and 12 ± 7 nM (n = 5) and $12 \text{ nM} \pm 2$ (n = 2, $\pm$ range), respectively, in the absence of PSD-95_{c-Myc}. Thus, by increasing the L-glutamate concentration to 1 mM, the affinity of NR1-1a/NR2A receptors for MK801 in the presence of PSD-95_{c-Myc} was increased to a level comparable to that of NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}.

Graphs A and B in Figure 3.15 show representative competition curves plotted with absolute specific [³H]MK801 binding (*i.e.* before normalization to percentage specific binding). These curves show that by increasing the concentration of L-glutamate to 1 mM, the specific binding of [³H]MK801 in the absence of added MK801 to NR1-1a/NR2A receptors + PSD-95_{c-Myc} was increased from 353 ± 5 (typical triplicate determination) to 567 ± 20 (typical triplicate determination) fmol [³H]MK801 binding sites/mg protein. This is a clear demonstration that the affinity of NR1-1a/NR2A receptors + PSD-95_{c-Myc} for MK801 was increased in the presence of 1 mM L-glutamate. The corresponding values for NR1-1a/NR2A receptor in the absence of PSD-95_{c-Myc} were 146 ± 8 and 142 ± 28 fmol [³H]MK801 binding sites/mg protein, illustrating that the affinity of MK801 was unchanged by this increase in the concentration of L-glutamate.

3.13 DETERMINATION OF THE AFFINITY OF NR1-1a/NR2A RECEPTORS $\pm$ PSD-95_{c-Myc} FOR L-GLUTAMATE AND GLYCINE

The effect of PSD-95_{c-Myc} on the sensitivity of [³H]MK801 binding to L-glutamate and glycine may be due to a decrease in the affinity of NR1-1a/NR2A receptors for L-glutamate and glycine. To investigate this possibility the affinity of NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} for both L-glutamate and glycine were determined using the glutamate site antagonist [³H]CGP 39653 and the glycine site antagonist [³H]MDL 105,519.

3.13.1 Characterisation of [³H]CGP 39653 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

Optimization of [³H]CGP 39653 binding to NR1-1a/NR2A receptors

A preliminary study was performed to determine the optimal conditions for [³H]CGP 39653 radioligand binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} expressed in HEK 293 cells. Cell homogenates were washed extensively according to the same method used in the preparation of cell homogenates for the L-glutamate and glycine enhancement of [³H]MK801 binding. Ideally, the method for the preparation of cell homogenates and the conditions of the radioligand binding assays would be identical for all radioligands used. Cell homogenates (100 µg protein) were assayed for the binding of [³H]CGP 39653 (6 nM) in the presence and absence of 10 µM 7-CK, using 100 µM L-glutamate for the determination of non-specific binding. The glycine site antagonist, 7-CK, was included to displace basal levels of glycine in the receptor preparation since the binding of [³H]CGP 39653 is negatively regulated by glycine (Mugnaini *et al.*, 1993). The results are summarised in Table 3.5. The specific [³H]CGP 39653 binding measured under either of these conditions was insufficient to perform accurate L-glutamate displacement studies. Therefore, the method of cell homogenate preparation and the conditions of the radioligand binding assay were modified, as described in 2.2.30.4, according to previously published methods for [³H]CGP 39653 binding to transfected HEK 293 cell homogenates (Kendrick *et al.*, 1996). The results for [³H]CGP 39653 (6 nM) binding in these conditions are summarised in Table 3.5. Specific [³H]CGP 39653 binding to NR1-1a/NR2A ± PSD-95_{c-Myc} was enhanced over ten-fold due to these methodological modifications. Furthermore, the inclusion of 10 µM 7-CK in the radioligand binding assays enhanced the specific [³H]CGP 39653 binding by approximately 2-fold. HEK 293 cell homogenates were therefore routinely prepared using this modified method and [³H]CGP 39653 binding was routinely measured in the presence of 10 µM 7-CK. Because glycine modulates [³H]CGP 39653 binding, and PSD-95_{c-Myc} modulates the glycine sensitivity of [³H]MK801 binding, any differential effect of glycine on the binding of [³H]CGP 39653 ± PSD-95_{c-Myc} would be minimized by the inclusion of the glycine site antagonist. The antagonist, 7-CK, was chosen because it has minimal allosteric effects on the other NMDA receptor recognition sites and acts purely to antagonise glycine binding (Kemp and Leeson, 1993; Grimwood *et al.*, 1993).

Saturation binding of [³H]CGP 39653 to NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

Prior to the determination of the affinity of NR1-1a/NR2A receptors ± PSD-95_{c-Myc} for L-glutamate by competition assay, the affinity of [³H]CGP 39653 for NR1-1a/NR2A receptors ± PSD-95_{c-Myc} was determined. Full saturation analyses were performed as described in 2.2.30.4. Representative curves are shown in Figure 3.17 and binding parameters are summarised in Table 3.6. [³H]CGP 39653 binding to NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc} was not significantly best fit by a two site binding model compared to a one site model in all cases (*i.e.* $p > 0.05$, F test). Analysis of the curves yielded K_D values of 6.6 ± 3.1 nM ($n = 3$) and 4.7 ± 0.6 nM ($n = 3$) in the presence and absence of PSD-95_{c-Myc} respectively. The corresponding B_{max} determinations were 1800 ± 200 ($n=3$) and 760 ± 120 ($n = 3$) fmol [³H]CGP 39653 binding sites/mg protein. Therefore, the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors significantly increased the number of [³H]CGP 39653 binding sites/mg protein ($n = 3$; $p < 0.005$, unpaired t test) while no significant change in the affinity of NR1-1a/NR2A receptors for [³H]CGP 39653 was observed.

3.13.2 Determination of the affinity of NR1-1a/NR2A RECEPTORS ± PSD-95_{c-Myc} for L-glutamate by displacement of [³H]CGP 39653

The affinity of NR1-1a/NR2A receptors for L-glutamate in the presence and absence of PSD-95_{c-Myc} was determined by the displacement of [³H]CGP 39653 (6 nM) binding by L-glutamate as described in 2.2.30.4. Representative competition curves are shown in Figure 3.18. The curves were best fit by a one-site binding model both in the presence and absence of PSD-95_{c-Myc} ($n = 3$; $p > 0.1$ and $p > 0.1$ respectively, F test) with Hill coefficients of 0.90 ± 0.05 ($n = 3$) and 1.03 ± 0.11 ($n = 3$) respectively. Corresponding K_i values were 71 ± 18 nM ($n = 3$) and 81 ± 13 nM ($n = 3$). Thus, PSD-95_{c-Myc} did not significantly alter the affinity of NR1-1a/NR2A receptors for L-glutamate.

3.13.3 Determination of the affinity of NR1-1a/NR2A receptors ± PSD-95_{c-Myc} for glycine by displacement of [³H]MDL 105,519

The affinity of NR1-1a/NR2A receptors for glycine in the presence and absence of PSD-95_{c-Myc} was determined by the displacement of [³H]MDL 105,519 by glycine as described in 2.2.30.2. The binding curve shown in Figure 3.19 is the combined data of the

competition assays performed from $n = 4$ independent transfections. Binding parameters are summarised in Table 3.6. Both curves were significantly best fit ($n = 4$; $P < 0.0001$, F test) by a two site binding model with Hill coefficients of 0.71 ± 0.07 ($n = 4$) and 0.70 ± 0.07 ($n = 4$) for NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} respectively. The K_D s determined for the high affinity site were $1.9 \pm 1.3 \mu\text{M}$ ($n = 4$) and $1.9 \pm 1.0 \mu\text{M}$ ($n = 4$) in the presence and absence of PSD-95_{c-Myc} respectively. The corresponding K_D s for the lower affinity site were $30 \pm 19 \mu\text{M}$ and $34 \pm 17 \mu\text{M}$ respectively. PSD-95_{c-Myc} did not therefore, significantly alter the affinity of glycine for either the high affinity or the low affinity binding sites. The fraction of the high affinity binding sites was $49 \pm 15 \%$ and $56 \pm 16 \%$ in the presence and absence of PSD-95_{c-Myc} respectively, demonstrating that PSD-95_{c-Myc} did not alter the percentage contribution of each binding site.

3.14 CHARACTERISATION OF THE NR2A^{Trunc} AND NR2B^{Trunc} MUTANTS

To establish whether the observed effects of PSD-95 on the expression of NMDA receptors in HEK 293 cells and on their radioligand binding properties were due to a specific interaction between the NR2 subunits and PSD-95, C-terminally truncated NR2A and NR2B subunits were generated as described in detail in 2.2.16. The region of both NR2 subunits thought to mediate an interaction with the PDZ domains of PSD-95 is a consensus tSXV motif located at the very end of the NR2 C-termini. Mutagenesis of either the valine at amino acid position 0 or the serine at position -2 abolished the interaction of the Kv1.4 K⁺ channel with PSD-95 (Kim *et al.*, 1995). Therefore, mutagenesis was used to generate NR2A and NR2B C-terminal truncation mutants lacking their C-terminal ESDV motifs.

Initial experiments were performed to characterise NR2A^{Trunc} and NR2B^{Trunc} with respect to their expression in HEK 293 cells, their ability to form functional NMDA receptors and their [³H]MK801 binding properties.

3.14.1 Quantitative analysis of the expression of NR2A^{Trunc} and NR2B^{Trunc} subunits in HEK 293 cells

To compare the expression level of the NR2A^{Trunc} protein with wild-type NR2A (NR2A^{WT}), HEK 293 cells were transfected either NR2A^{WT} or NR2A^{Trunc}, and NR1-1a/NR2A^{WT} or NR1-1a/NR2A^{Trunc} receptor clones. The resulting cell homogenates

were analysed by quantitative immunoblotting using the anti-NR2A/2B (1,435-1,445) and anti-NR2A (1,381-1,394) antibodies. Representative immunoblots are shown in Figure 3.20. Anti-NR2A (1,381-1,394) antibodies labelled a single polypeptide in cells transfected with NR2A^{WT} or NR2A^{Trunc} with $M_r = 180$ kDa, corresponding to the NR2A subunit. Surprisingly, the anti-NR2A/2B (1,435-1,445) antibody did not label any immunoreactivity from cells transfected with NR2A^{Trunc}. A possible explanation for this is that the four C-terminal amino acids truncated from the NR2A subunit to generate NR2A^{Trunc} were contained in the peptide, YKKMPSIESDV, used to generate the anti-NR2A/2B (1,435-1,445) antibodies. Therefore, the removal of these four amino acids must have been sufficient to abolish the antigenicity of the NR2A^{Trunc} subunit to these antibodies.

Quantitative immunoblotting revealed that when expressed alone the expression level of the NR2A^{WT} subunit was 6.8-fold ± 0.5 ($n = 2$, $\pm$ range) higher compared to the NR2A^{Trunc} subunit. However, when cotransfected with NR1-1a, the ratio of the intensity of NR2A^{WT} immunoreactivities to the intensity of NR2A^{Trunc} immunoreactivities was 1.2 ± 0.4 ($n = 5$). When expressed with an NR1-1a subunit therefore, the expression of the NR2A^{Trunc} subunit was not significantly different from NR2A^{WT}.

The immunoblots corresponding to an identical characterisation carried out on the NR2B^{Trunc} subunit are shown in Figure 3.21. The anti-NR2B (46-60) antibody detected a single polypeptide of $M_r = 180$ kDa in HEK 293 cells transfected with NR2B^{Trunc}. Again the anti-NR2A/2B (1,435-1,445) antibody did not detect the $M_r = 180$ kDa NR2B^{Trunc} subunit indicating that truncation of the ESDV amino acids from the NR2B subunit also abolished the antigenicity of the NR2B^{Trunc} subunit. Quantitative immunoblotting revealed that when expressed alone or in NR1/NR2 subunit combinations NR2B^{WT} and NR2B^{Trunc} were expressed to a similar level. The ratio of NR2B^{WT} immunoreactivities to NR2B^{Trunc} was 1.1 ($n = 1$) and 1.2 ($n = 1$) when expressed alone and with an NR1-1a subunit respectively.

3.14.2 Functional characterisation of NR1-1a/NR2A^{Trunc} and NR1-1a/NR2B^{Trunc} receptors

The expression of functional NMDA receptors composed of truncated NR2 subunits was investigated by assaying the NMDA receptor-mediated cell death following transfection of HEK 293 cells with either NR1-1a/NR2A^{WT} or NR1-1a/NR2A^{Trunc}, and NR1-1a/NR2B^{WT} or NR1-1a/NR2B^{Trunc} receptor clones. Co-expression of NR1-1a/NR2A^{WT}

and NR1-1a/NR2A^{Trunc} resulted in 22 ± 3 % ($n = 3$) and 23 ± 2 % ($n = 2 \pm \text{range}$) cell death respectively. Co-expression of NR1-1a/NR2B^{WT} and NR1-1a/NR2B^{Trunc} receptors resulted in 25 ± 3 % ($n = 2, \pm \text{range}$) and 23 ± 1 % ($n = 2, \pm \text{range}$) cell death respectively. The percentage cell death resulting from the expression of NMDA receptors composed of truncated NR2 subunits was therefore comparable to that obtained with wild-type NR2 subunits, confirming the co-assembly of both truncated subunits with NR1-1a to form functional NMDA receptors.

The NR2A^{Trunc} subunit was further characterised by radioligand binding to establish whether NR1-1a/NR2A^{Trunc} receptors behaved as wild-type receptors with respect to their ability to bind [³H]MK801 with high affinity. Full saturation assays were performed on NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors in the presence of 1 mM L-glutamate. The resulting binding parameters are summarised in Table 3.7. Both NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors displayed similar affinities for [³H]MK801, with corresponding K_D values of 12 ± 7 (n = 5) and 13 ± 1 nM (n = 3), and B_{max} values of 680 ± 60 (n = 3) and 570 ± 70 fmol [³H]MK801 binding sites/mg protein (n = 4) respectively. The number of [³H]MK801 binding sites was not significantly different between the wild-type and the truncated form of receptor, supporting the observation that both NR2A^{Trunc} and NR2A^{WT} subunits were expressed to a similar level when co-expressed with NR1-1a.

In order to confirm that the binding of [³H]MK801 to both NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors was equally sensitive to allosteric modulation by L-glutamate, full dose-response curves for the enhancement of [³H]MK801 binding by L-glutamate were performed. The resulting EC_{50} values of 1.8 ± 0.4 μM (n = 4) and 1.5 μM (n = 1) for wild type and truncated receptors respectively, were not significantly different. Thus, in summary, the NR2A^{Trunc} mutants and NR2^{WT} subunits displayed identical properties with respect to their expression with NR1-1a subunits, their association with NR1-1a subunits to form functional NMDA receptors, their affinity for [³H]MK801, and their sensitivity to modulation by L-glutamate.

3.15 QUANTITATIVE ANALYSIS OF THE EXPRESSION OF NR1-1a/NR2 TRUNCATION MUTANTS $\pm$ PSD-95_{c-Myc}

To assess whether the specific increase in NR2 subunit immunoreactivities induced by the co-expression of PSD-95_{c-Myc} was dependent upon an interaction between

PSD-95_{c-Myc} and the C-terminal tails of the NR2 subunits, NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors were expressed in HEK 293 cells both in the presence and absence of PSD-95_{c-Myc}. Resulting cell homogenates were analysed by quantitative immunoblotting using anti-NR2A (1,381-1394), anti-NR1 (911-920) and anti-c-Myc antibodies. Representative immunoblots are shown in Figure 3.22. Consistent with previous experiments (as described in section 3.3), PSD-95_{c-Myc} induced a 2.5 ± 0.4 -fold ($n = 5$) increase in the intensity of the NR2A^{WT} immunoreactivity, with no significant change in the NR1-1a subunit. However, this increase in NR2 immunoreactivities was not seen when PSD-95_{c-Myc} was co-expressed with NR1-1a/NR2A^{Trunc}. The ratio of NR2A^{Trunc} immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to intensities measured in the absence of PSD-95_{c-Myc} was 0.6 ± 0.2 ($n = 6$). The increase in NR2A^{Trunc} immunoreactivities induced by PSD-95_{c-Myc} was therefore abolished by the C-terminal truncation of the NR2A subunit. In fact, a slight decrease in the intensity of the NR2A^{Trunc} subunit immunoreactivity was observed in the presence of PSD-95_{c-Myc}. The ratios of NR1-1a immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to the intensities measured in the absence of PSD-95_{c-Myc} were 1.1 ± 0.2 ($n = 4$) for NR1-1a/NR2A^{WT} and 1.0 ± 0.4 ($n = 7$) for NR1-1a/NR2A^{Trunc}. With respect to the NR1-1a subunit therefore, no significant change in the intensities of NR1-1a immunoreactivities was induced by the co-expression of PSD-95_{c-Myc} whether NR1-1a was expressed with NR2A^{WT} or NR2A^{Trunc}.

The effect of the NR2B subunit C-terminal truncation on the PSD-95_{c-Myc}-mediated increase in NR2 subunit immunoreactivities was also characterised. Either NR1-1a/NR2B^{WT} or NR1-1a/NR2B^{Trunc} receptors were expressed in HEK 293 cells in the presence and the absence of PSD-95_{c-Myc}, and the resulting cell homogenates were assayed for the expression of NR1-1a and NR2B subunits by quantitative immunoblotting. Consistent with results obtained in section 3.3, the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2B^{WT} resulted in a 2.2 ± 0.8 -fold ($n = 3$) increase in the intensity of the NR2B subunit immunoreactivity. However, the ratio of NR2B^{Trunc} immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to intensities measured in the absence of PSD-95_{c-Myc} was 0.8 ± 0.4 ($n = 3$). Corresponding ratios for the effect of PSD-95_{c-Myc} on the NR1-1a subunit expression were 1.0 ± 0.3 ($n = 3$) for NR1-1a/NR2B^{WT} and 1.0 ± 0.3 ($n = 3$) for NR1-1a/NR2B^{Trunc}. The effect of PSD-95_{c-Myc} on the intensity of the NR2B subunit immunoreactivity was therefore abolished by this C-terminal truncation, while no change was observed in the expression level of NR1-1a subunit. The immunoblot shown

in Figure 3.22 illustrates that intensity of the NR2B^{Trunc} subunit immunoreactivity in the presence of PSD-95_{c-Myc} was comparable to the intensity of the NR2B^{WT} immunoreactivity in the absence of PSD-95_{c-Myc}.

Interestingly both the anti-NR2A (1,381-1,394) and anti-NR2A/2B (1,435-1,445) antibodies sometimes labelled a doublet with $M_r = 180$ kDa and $M_r = 175$ kDa in HEK 293 cells expressing either NR2A or NR2B (see Figure 3.22 A, lane 5). In those immunoblots where the lower molecular weight species was detectable, only a single NR2A or NR2B immunoreactive species of $M_r = 180$ kDa was detected when the NR2 subunit was co-expressed with PSD-95_{c-Myc}. In addition, the anti-NR2A (1,381-1,394) antibody identified the same doublet in cells expressing NR1-1a/NR2A^{Trunc}. The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A^{Trunc} again abolished the lower molecular weight band (Figure 3.22 A, lane 7). If this effect of PSD-95_{c-Myc} is dependent upon an interaction with the NR2A subunit, then it would appear that PSD-95_{c-Myc} may in fact interact with NR2A^{Trunc}.

A similar phenomenon was sometimes observed with anti-NR1 (911-920) antibodies. As shown in Figure 3.5, these antibodies occasionally detected an additional immunoreactive band with $M_r = 95$ kDa. When the NR1-1a/NR2A^{WT} or NR1-1a/NR2A^{Trunc} receptors were co-expressed with PSD-95_{c-Myc} this lower molecular weight band was not detected.

3.16 THE EFFECT OF THE NR2A C-TERMINAL TRUNCATION ON [³H]MK801 SATURATION BINDING TO NR1-1a/NR2A RECEPTORS ± PSD-95_{c-Myc}

As described in section 3.15, the deletion of the last four amino acids from both the NR2A and NR2B subunit C-termini abolished the PSD-95_{c-Myc}-mediated increase in the intensity of NR2 subunit immunoreactivities. The analysis of transfected cell homogenates by quantitative immunoblotting did not however reveal whether these mutations abolished the effect of PSD-95_{c-Myc} on the level of assembled receptor. Therefore, HEK 293 cells were transfected with NR1-1a/NR2A^{WT} or NR1-1a/NR2A^{Trunc} receptor clones ± PSD-95_{c-Myc} and the resulting cell homogenates were analysed by [³H]MK801 saturation binding in the presence of 1 mM L-glutamate. Representative binding curves with the corresponding binding parameters are shown in Figure 3.23. In all cases the binding curves were best-fit by a one site binding model. The B_{max} values for [³H]MK801 binding to NR1-1a/NR2A^{WT}

receptors in the presence and absence of PSD-95_{c-Myc} were 2200 ± 400 ($n = 4$) and 680 ± 60 ($n = 4$) fmol [³H]MK801 binding sites/mg protein respectively. In agreement with results obtained in section 3.6, PSD-95_{c-Myc} induced a significant 3.2 ± 0.3 -fold ($n = 4$; $p < 0.005$, paired t test) increase in the $B_{\max}$ values for [³H]MK801 binding to NR1-1a/NR2A^{WT} receptors. However, the truncation of the NR2A C-terminal ESDV motif significantly reduced this PSD-95_{c-Myc}-induced increase in the level of assembled receptor. The $B_{\max}$ values for [³H]MK801 binding to NR1-1a/NR2A^{Trunc} receptors in the presence and absence of PSD-95_{c-Myc} were 880 ± 110 ($n = 3$) and 570 ± 70 ($n = 3$) fmol [³H]MK801 binding sites/mg protein respectively. Thus, although the majority of the increase in the number of [³H]MK801 binding sites induced by PSD-95_{c-Myc} was abolished by truncating the C-terminus of NR2A, PSD-95_{c-Myc} still induced a 57 ± 24 % ($n = 3$; $p < 0.05$, paired t test) increase in the number of [³H]MK801 binding sites/mg protein. The significance of this increase in NR1-1a/NR2A^{Trunc} receptor binding sites suggested that PSD-95_{c-Myc} may still associate with the NR2A^{Trunc} subunit but perhaps to a lesser extent than with NR2A^{WT}. This possibility was subsequently addressed in section 3.22.3.

It was expected that the saturation analysis in the presence of 1 mM L-glutamate would yield K_D values that were comparable between all four transfection conditions since the change in the affinity for MK801 $\pm$ PSD-95_{c-Myc} observed initially was shown to be due to a change in sensitivity to L-glutamate, rather than a change in the affinity for MK801. However, in contrast to the apparent K_i values determined in the presence of 1 mM L-glutamate, PSD-95_{c-Myc} induced a decrease ($n = 3$; $p < 0.05$, unpaired t test) in the affinity of NR1-1a/NR2A^{WT} receptors for [³H]MK801 as determined by the saturation binding in the presence of 1 mM L-glutamate. The K_D s determined were 25 ± 4 ($n = 5$) and 17 ± 5 ($n = 5$) for NR1-1a/NR2A^{WT} receptors in the presence and absence of PSD-95_{c-Myc} respectively. This effect of PSD-95_{c-Myc} on the affinity of NR1-1a/NR2A^{WT} receptors for [³H]MK801, even in the presence of 1 mM L-glutamate, was not observed for NR1-1a/NR2A^{Trunc} receptors. Saturation analysis yielded K_D s of 12 ± 1 ($n = 3$) and 13 ± 1 ($n = 3$) in the presence and absence of PSD-95_{c-Myc} respectively.

3.17 ALLOSTERIC MODULATION OF [³H]MK801 BINDING TO NR1-1a/NR2A^{Trunc} RECEPTORS $\pm$ PSD-95_{c-Myc}

The mechanism by which PSD-95_{c-Myc} enhanced the level of NR1-1a/NR2A receptors in transfected HEK 293 cells may have been independent of the mechanism by

which PSD-95_{c-Myc} decreased the sensitivity of NR1-1a/NR2A receptors to L-glutamate as measured by the enhancement of [³H]MK801 binding by L-glutamate. To investigate whether the effect of PSD-95_{c-Myc} on the L-glutamate sensitivity of NR1-1a/NR2A receptors was indeed dependent upon an interaction between NR2 subunits and PSD-95_{c-Myc}, the L-glutamate sensitivity of NR1-1a/NR2A^{Trunc} receptors was assayed in the presence and absence of PSD-95_{c-Myc}.

Consistent with the expected decrease in the L-glutamate sensitivity of NR1-1a/NR2A^{WT} receptors in the presence of PSD-95_{c-Myc}, the enhancement ratios (summarised in Figure 3.24) for the L-glutamate modulation of [³H]MK801 binding determined in the presence and absence of PSD-95_{c-Myc} were 1.57 ± 0.21 (n = 11) and 1.00 ± 0.08 (n = 13) respectively. The corresponding determinations for NR1-1a/NR2A^{Trunc} receptors revealed that this change in sensitivity to L-glutamate was completely dependent upon the NR2A C-terminal ESDV motif. The enhancement ratios for NR1-1a/NR2A^{Trunc} receptors in the presence and absence of PSD-95_{c-Myc} were 1.08 ± 0.06 (n = 4) and 1.06 ± 0.03 (n = 4) respectively.

3.18 CHARACTERISATION OF THE PSD-95_{c-Myc}(C3S/C5S) MUTANT

The PSD-95_{c-Myc}(C3S/C5S) mutant was generated to investigate whether the observed effects of PSD-95 on the expression level of NMDA receptors in HEK 293 cells and on their pharmacological properties were dependent upon the clustering function of PSD-95. The two N-terminal cysteine residues located at amino acid positions 3 and 5 of PSD-95 are required for the formation of receptor clusters upon co-expression of PSD-95 with shaker type K⁺ channels and NMDA receptors in heterologous cells (Kim *et al.*, 1995; Hsueh *et al.*, 1997). PSD-95 constructs lacking these two cysteine residues were unable to recruit NMDA receptors into receptor rich domains even though the association of the NR2 subunit with PSD-95 was maintained (Hsueh *et al.*, 1997). The precise mechanism of this clustering has not been fully investigated, although Hsueh and Sheng, (1999) propose that PSD-95 molecules are able to co-assemble via the formation of disulphide bridges between their N-terminal cysteine residues. In contrast, El-Husseini *et al.* (2000a) propose that dual palmitoylation of these two cysteines is responsible for ion channel clustering.

The clustering deficient PSD-95_{c-Myc} mutant was generated as described in detail in 2.2.16. The two N-terminal cysteines were substituted for serine residues by *in vitro* site-directed mutagenesis. Serine and cysteine are both uncharged amino acids with polar side

chains, and only differ by the presence of a hydroxyl group side chain in serine compared to a sulphhydryl group in cysteine. The exchange of cysteine with serine was therefore the most appropriate amino acid substitution.

A preliminary characterisation was carried out to verify that this mutant form of PSD-95 was expressed to the same level as the wild type. HEK 293 cells were transfected with either wild-type PSD-95_{c-Myc} or PSD-95_{c-Myc}(C3S/C5S) and cell homogenates were analysed by quantitative immunoblotting. Initial experiments showed that the expression of PSD-95_{c-Myc} was reduced by the substitution of the two N-terminal cysteine residues. A possible explanation for the difference in the expression level determined by immunoblot analysis of cell homogenates is that the PSD-95_{c-Myc}(C3S/C5S) may be differentially distributed between the membrane and cytoplasmic cellular fractions. PSD-95 does not display any putative transmembrane domains and is therefore considered to be a soluble cytoplasmic protein. However, it has been shown that PSD-95 can associate with the plasma membrane via the lipid modification of its two cysteine residues located at amino acid positions 3 and 5 (Topinka and Bredt, 1998). The mutation of these two cysteine residues may therefore result in a reduced ability of PSD-95_{c-Myc}(C3S/C5S) to associate with the plasma membrane. The transfected cell homogenates prepared for analysis by immunoblotting are processed by a series of homogenisation and centrifugation steps, consequently removing the majority of the cytoplasmic proteins. If PSD-95_{c-Myc}(C3S/C5S) was preferentially distributed to the cytoplasm compared to wild-type PSD-95_{c-Myc}, then the immunoblot analysis of cell homogenates may indicate a lower expression level of PSD-95_{c-Myc}(C3S/C5S).

To test this hypothesis, the pellets and supernatants of the transfected cells were collected after a single cycle of homogenisation and centrifugation. These were then analysed by quantitative immunoblotting to investigate the relative distribution of PSD-95_{c-Myc}(C3S/C5S) and PSD-95_{c-Myc} between the membrane and cytoplasmic fractions. As shown in Figure 3.25, the intensity of the PSD-95_{c-Myc}(C3S/C5S) immunoreactivity detected in the pellet was reduced to $59 \pm 18 \%$ ($n = 6$; $p < 0.005$, paired t test) of the corresponding intensity of PSD-95_{c-Myc}. However, this difference was not observed in the supernatants where the intensity of PSD-95_{c-Myc}(C3S/C5S) immunoreactivity was $115 \pm 5 \%$ ($n = 2$, $\pm$ range) of the corresponding PSD-95_{c-Myc} intensity, implying that PSD-95_{c-Myc}(C3S/C5S) was redistributed to the cytoplasmic fraction. The total expression of the PSD-95_{c-Myc}(C3S/C5S) mutant, *i.e.* in the pellet plus the supernatant, was reduced by $16 \% \pm 7$ ($n = 2$, $\pm$ range) compared to the wild-type PSD-95_{c-Myc} suggesting that the two

N-terminal mutations did not significantly decrease the expression of PSD-95_{c-Myc} in HEK 293 cells.

An additional experiment was performed to investigate whether the formation of disulphide bonds between the N-terminal cysteine residues of PSD-95 was responsible for the homo-multimerization of PSD-95. Following the transfection of HEK 293 cells with PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) the cells were harvested and homogenised. The resulting cell homogenates were then analysed by immunoblotting under non-reducing conditions, *i.e.* samples were prepared for SDS-PAGE without the addition of DTT. As shown in Figure 3.26, the anti-c-Myc antibody labelled two faint immunoreactive bands with $M_r = 180$ kDa and 300-350 kDa in cells expressing PSD-95_{c-Myc} in addition to the band, $M_r = 100$ kDa, corresponding to monomeric PSD-95_{c-Myc}. In reducing conditions these bands were no longer detected. In addition, they were not observed in HEK 293 cells expressing PSD-95_{c-Myc}(C3S/C5S) either under reducing or non-reducing conditions. The molecular weights of these two extra bands are roughly equivalent to dimers and tetramers of PSD-95_{c-Myc} suggesting a possible multimerization of PSD-95_{c-Myc} molecules under non-reducing conditions that is dependent upon the N-terminal cysteine residues. However, the vast majority of the PSD-95_{c-Myc} immunoreactivity was present as a 100 kDa band.

3.19 THE EFFECT OF PSD-95_{c-Myc}(C3S/C5S) ON THE EXPRESSION LEVEL OF NR1-1a/NR2A RECEPTORS

To investigate whether the clustering function of PSD-95_{c-Myc} was required for the specific increase in NR2A subunit expression, the effects of PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) on the level of NR1-a/NR2A receptor expression in HEK 293 cells were compared.. Consistent with results obtained in section 3.3 the analysis of the resulting cell homogenates by quantitative immunoblotting revealed that PSD-95_{c-Myc} induced a 2.2 ± 0.2 -fold ($n = 3$; $p < 0.01$, paired t test) increase in the intensity of NR2A subunit immunoreactivities (see Figure 3.27). However, this effect of PSD-95_{c-Myc} on the level of NR2A subunit expression was significantly ($n = 3$, $p < 0.01$, unpaired t test) diminished but not completely abolished by mutating the two N-terminal cysteine residues to serine. As shown in Figure 3.27, PSD-95_{c-Myc}(C3S/C5S) induced a 1.3 ± 0.2 -fold ($n = 3$) increase in the intensity of the NR2A immunoreactivity. The level of NR1-1a remained unchanged by the co-expression of NR1-1a/NR2A receptors with either PSD-95_{c-Myc} or PSD-95_{c-Myc}(C3S/C5S). The ratios of NR1-1a immunoreactive intensities measured in the

presence of either of PSD-95_{c-Myc} or PSD-95_{c-Myc}(C3S/C5S) to intensities measured in the absence of PSD-95_{c-Myc} were 1.1 ± 0.2 ($n = 3$) for PSD-95_{c-Myc} and 0.96 ± 0.1 ($n = 3$) for PSD-95_{c-Myc}(C3S/C5S).

3.20 THE EFFECT OF PSD-95_{c-Myc}(C3S/C5S) ON THE [³H]MK801 BINDING PROPERTIES OF NR1-1a/NR2A RECEPTORS

The effect of the C3S/C5S mutations on the ability of PSD-95_{c-Myc} to alter the binding properties of NR1-1a/NR2A receptors was investigated with respect to the PSD-95_{c-Myc}-induced increase in the number of [³H]MK801 binding sites and the decrease in the sensitivity of [³H]MK801 binding to L-glutamate.

Single point [³H]MK801 binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}(C3S/C5S)

Single point binding [³H]MK801 (40 nM) binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} and in the presence of PSD-95_{c-Myc}(C3S/C5S) were 1200 ± 80 ($n = 3$), 420 ± 60 ($n = 3$) and 790 ± 100 ($n = 3$) fmol [³H]MK801 binding sites/mg protein respectively (summarised in Figure 3.28). Consistent with the characterised effect of PSD-95_{c-Myc} on the [³H]MK801 binding properties of NR1-1a/NR2A receptors, PSD-95_{c-Myc} induced a significant 3.5 ± 0.2 -fold increase ($n = 3$; $p < 0.005$, paired t test) in the number of [³H]MK801 binding sites/mg protein. Single point determinations revealed that this increase was significantly reduced ($n = 3$, $p < 0.05$, paired t test) but not abolished in the presence of PSD-95_{c-Myc}(C3S/C5S). The number of [³H]MK801 binding sites/mg protein was enhanced by 2.4 ± 0.1 fold ($n = 3$, $p < 0.005$ paired t test) in the presence of PSD-95_{c-Myc}(C3S/C5S). The substitution of the two N-terminal cysteine residues therefore reduced the PSD-95_{c-Myc}-induced increase in the number of [³H]MK801 binding sites in cells expressing NR1-1a/NR2A receptors to an intermediate value. To confirm that the PSD-95_{c-Myc}(C3S/C5S) mutant did not alter the affinity of NR1-1a/NR2A receptors for [³H]MK801 to an extent that may explain the change in single point [³H]MK801 binding, full saturation [³H]MK801 binding to NR1-1a/NR2A/PSD-95_{c-Myc}(C3S/C5S) receptors was carried out. The resulting K_D of 24 nM was very close to value of 25 ± 4 nM ($n = 5$) obtained for [³H]MK801 binding to NR1-1a/NR2A/PSD-95_{c-Myc} receptors and thus, the single point binding data was an acceptable measure of the number of assembled NR1-1a/NR2A receptors.

Modulation of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} (C3S/C5S) by L-glutamate

The L-glutamate sensitivity of [³H]MK801 binding to NR1-1a/NR2A^{WT} receptors in the presence and absence of PSD-95_{c-Myc} and in the presence of PSD-95_{c-Myc}(C3S/C5S) was determined as described in section 3.8. The results are summarised in Figure 3.29. As predicted for the PSD-95-mediated decrease in sensitivity to L-glutamate, the ratios of the percentage enhancement of [³H]MK801 binding at 1 mM L-glutamate to the enhancement determined at 10 µM L-glutamate were 1.57 ± 0.21 ($n = 11$) and 1.00 ± 0.08 ($n = 13$) in the presence and absence of PSD-95_{c-Myc} respectively. However, the substitution of the two N-terminal cysteines in PSD-95_{c-Myc} significantly ($n = 3$; $p < 0.05$, unpaired t test) reduced the effect of PSD-95_{c-Myc} on the L-glutamate sensitivity of [³H]MK801 binding, although the PSD-95_{c-Myc}(C3S/C5S) mutant still significantly ($n = 3$; $p < 0.005$) increased the enhancement ratio above unity, to an intermediate value of 1.25 ± 0.10 ($n = 3$). PSD-95_{c-Myc}(C3S/C5S) therefore reduced the sensitivity of NR1-1a/NR2A receptors to L-glutamate but to a lesser extent compared to the wild-type PSD-95_{c-Myc}.

3.21 CO-EXPRESSION OF PSD-95_{c-Myc} WITH NMDA RECEPTORS COMPOSED OF ALTERNATIVELY SPLICED NR1 SUBUNITS

The alternatively spliced exons of the NR1 gene are thought to be involved in the regulation of the cell surface expression of the NR1 subunit in transfected hippocampal neurons (Ehlers *et al.*, 1995; Okabe *et al.*, 1999a). Therefore, even though PSD-95_{c-Myc} did not alter the expression level of the NR1-1a subunit in transfected HEK 293 cells, as defined by quantitative immunoblotting and [³H]MDL 105,519 binding, the NR1-1a subunit may be critical in the control of receptor expression and turnover. This possible role of the NR1-1a may be dependent upon the specific exons contained within the NR1-1a splice variant and thus, the ability of PSD-95_{c-Myc} to enhance the expression level of functional NMDA receptors in HEK 293 cells may be altered by changing the NR1 splice variant. Consequently, the effect of exchanging the NR1-1a splice variant for the alternatively spliced NR1-2a and NR1-4b subunits on the PSD-95_{c-Myc}-mediated increase in the expression of NR1/NR2A receptors was investigated. The NR1-1a, NR1-2a and NR1-4b splice variants were chosen because they represent the full complement of alternatively spliced exons of the NR1 gene, *i.e.* NR1-1a contains the C1 and C2 exons, NR1-2a contains the C2 exon, and NR1-4b contains the N1 and C2' exon.

3.21.1 The effect of the NR1 subunit splice form on the expression level of NMDA receptors $\pm$ PSD-95_{c-Myc}

Analysis of the expression of NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors in HEK 293 cells by quantitative immunoblotting

HEK 293 cells were transfected with either NR1-1a/NR2A, NR1-2a/NR2A or NR1-4b/NR2A. Cell homogenates were analysed by quantitative immunoblotting using anti-NR1 (17-35), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies. The anti-NR1 (17-35) antibodies were used because they were raised against a peptide that is present in all NR1 splice variants and therefore label the different splice forms with equal affinity. As shown in Figure 3.30, the anti-NR1 (17-35) antibodies recognised a single polypeptide with $M_r = 117$ kDa from cells transfected with NR1-1a/NR2A receptor clones, consistent with the M_r of N-glycosylated NR1-1a protein (Chazot *et al.*, 1992). The M_r of polypeptides detected from cells transfected with NR1-2a/NR2A and NR1-4b/NR2A receptor clones were both approximately 115 kDa due to the absence of the 22 amino acid C1 exon in the NR1-2a and NR1-4b splice variants. Initial experiments analysed the level of the NR1-1a, NR1-2a and NR1-4b splice variants and the NR2A subunit expressed in NR1/NR2A subunit combinations by quantitative immunoblotting. There was no significant difference in the intensity of the NR2A subunit immunoreactivities when expressed with each NR1 splice variant. However, the ratios of the intensities of the NR1-2a and NR1-4b subunit immunoreactivities to the intensity of the NR1-1a subunit immunoreactivity were 0.6 ± 0.2 ($n = 3$; $p < 0.05$, paired t test) and 0.3 ± 0.1 ($n = 3$; $p < 0.005$, paired t test) respectively. The NR1-2a and NR1-4b splice variants were therefore expressed to a significantly lower level than the NR1-1a splice variant in NR1/NR2A subunit combinations.

The effect of PSD-95_{c-Myc} on the expression level of NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors by quantitative immunoblotting

HEK 293 cells were transfected with either NR1-1a/NR2A $\pm$ PSD-95_{c-Myc}, NR1-2a/NR2A $\pm$ PSD-95_{c-Myc} or NR1-4b/NR2A $\pm$ PSD-95_{c-Myc} and cell homogenates were analysed by immunoblotting using anti-NR1 (17-35), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies. Surprisingly, in addition to the detection of each NR1 splice variant, the anti-NR1 (17-35) antibodies detected a band with $M_r = 100$ kDa that was only present in HEK 293 cells transfected with PSD-95_{c-Myc} suggesting that this antibody cross reacted

with PSD-95_{c-Myc}. Cross reactivity was also found against the wild-type PSD-95 proteins. The ratios of the immunoreactive intensities of each NR1 splice variant and NR2A subunit measured in the presence of PSD-95_{c-Myc} to the intensities measured in the absence of PSD-95_{c-Myc} are summarised in Figure 3.30. Consistent with results obtained in section 3.3, the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors resulted in a 2.5 ± 0.5 -fold ($n = 3$) increase in the NR2A subunit immunoreactivity, with no concomitant change in the NR1-1a subunit. Co-expression of PSD-95_{c-Myc} with NR1-2a/NR2A and NR1-4b/NR2A receptors resulted in a 2.1 ± 0.4 -fold ($n = 3$) and a 2.9 ± 0.7 -fold ($n = 3$) increase in NR2A subunit immunoreactivities respectively. The PSD-95_{c-Myc}-induced increase in the expression of the NR2A subunit was therefore independent of the NR1 N1, C1 and C2 exons.

In contrast to the results obtained in section 3.3, where PSD-95_{c-Myc} did not effect the expression level of the NR1-1a splice variant, the co-expression of PSD-95_{c-Myc} with NR1-2a/NR2A and NR1-4b/NR2A receptors resulted in a 1.2 ± 0.2 -fold ($n = 3$) and a 1.8 ± 0.1 -fold ($n = 3$; $p < 0.05$, paired t test) increase in the intensity of NR1-2a and NR1-4b immunoreactivities respectively. The NR1-4b subunit was therefore expressed at a significantly higher level when co-expressed in an NR1/NR2A subunit combination in the presence of PSD-95_{c-Myc} compared to the control transfections of NR1-4b/NR2A in the absence of PSD-95_{c-Myc}. PSD-95_{c-Myc} may also enhance the expression level of the NR1-2a subunit immunoreactivity when co-expressed with NR1-2a/NR2A. However, the quantitative immunoblotting procedure was not sufficiently sensitive to detect the magnitude of this change to any level of significance.

Single point [³H]MK801 binding to NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors expressed in HEK 293 cells $\pm$ PSD-95_{c-Myc}

Initial single point [³H]MK801 binding assays were performed to determine whether the number of [³H]MK801 binding sites/mg protein detected in HEK 293 cells transfected with NR1/NR2A receptors was regulated by the NR1 subunit splice form. Cell homogenates expressing either NR1-1a/NR2A, NR1-2a/NR2A or NR1-4b/NR2A receptors were assayed for single point [³H]MK801 binding (40 nM) in the presence of 1 mM L-glutamate and 30 μ M glycine. As shown in Figure 3.31, the NR1 subunit splice form influenced the number of specific [³H]MK801 binding sites/mg protein detected in HEK 293 cells transfected with NR1/NR2A. The specific [³H]MK801 binding to NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors was 460 ± 90 ($n = 3$), 560 ± 140 ($n = 3$) and

790 ± 160 (n = 3) fmol [³H]MK801 binding sites/mg protein respectively. These results demonstrate that assembled NR1-4b/NR2A receptors were expressed at a significantly (n = 3, *p* < 0.05, paired *t* test) higher level than assembled NR1-1a/NR2A receptors, whereas assembled NR1-2a/NR2A receptors were expressed at an intermediate level.

Further single point [³H]MK801 radioligand binding assays were performed to assess whether the NR1 subunit splice form influenced the PSD-95_{c-Myc}-mediated increase in [³H]MK801 binding to HEK 293 cells transfected with NR1/NR2A receptors. Consistent with results obtained in section 3.6, PSD-95_{c-Myc} induced a 3.1 ± 0.6-fold (n = 3) increase in the specific [³H]MK801 binding to NR1-1a/NR2A receptors. However, this effect of PSD-95_{c-Myc} was reduced when NR2A subunits were co-assembled with either the NR1-2a or NR1-4b splice variants. Co-expression of PSD-95_{c-Myc} with NR1-2a/NR2A and NR1-4b/NR2A receptors resulted in a 2.0 ± 0.3-fold (n = 3, *p* < 0.05, paired *t* test) and 1.4 ± 0.2-fold (n = 3, *p* < 0.05, paired *t* test) increase in the number of [³H]MK801 binding sites/mg protein respectively. The PSD-95_{c-Myc}-mediated increase in the specific [³H]MK801 binding was therefore significantly reduced in the presence of the NR1-2a (n = 3, *p* < 0.05, unpaired *t* test) and NR1-4b (n = 3, *p* < 0.001, unpaired *t* test) splice variants.

To confirm whether the effect of the NR1 subunit splice variant on the relative levels of single point [³H]MK801 binding was in fact due to an exon-dependent change in the affinity of NR1/NR2A receptors for [³H]MK801, saturation analyses of [³H]MK801 binding to NR1-1a/NR2A ± PSD-95_{c-Myc}, NR1-2a/NR2A ± PSD-95_{c-Myc} and NR1-4b/NR2A ± PSD-95_{c-Myc} receptors were performed. The resulting *K_D*s are summarised in table 3.8. The *K_D* values obtained for [³H]MK801 binding to receptors composed of each NR1 splice variant were not substantially different. The differences observed in the single point [³H]MK801 binding (40 nM) between receptors composed of each NR1 splice variant could not therefore be explained by any differences in their affinities for [³H]MK801 and were thus acceptable measures for the relative number of assembled NR1/NR2A receptors .

3.21.2 Is the effect of PSD-95_{c-Myc} on the L-glutamate sensitivity of [³H]MK801 binding to NR1-1a/NR2A receptors dependent upon the NR1 subunit splice form?

The effects of PSD-95 on the sensitivity of NR1-1a/NR2A receptors to L-glutamate and glycine as defined by the enhancement of [³H]MK801 binding by either

L-glutamate or glycine, may be due to a PSD-95 dependent change in the phosphorylation state of either the NR1-1a or the NR2A subunit. The role of phosphorylation in the regulation of NMDA receptor function has been well characterised. For example, the *in vivo* and *in vitro* activation of protein kinases and phosphatases has been shown to both potentiate and inhibit NMDA receptor function (detailed in sections 1.3 and 1.4). Recently, the association of PSD-95 with a variety of protein tyrosine kinases and phosphatases was reported (Tezuka *et al.*, 1999; Kawachi *et al.*, 1999). In particular, PSD-95 has been shown to enhance the phosphorylation state of NR2A subunits via its co-association with fyn tyrosine kinase (Tezuka *et al.*, 1999). In addition, Yamada *et al.*, (1999) reported that PSD-95 abolished the potentiation of NR1-1a/NR2B receptor mediated currents by protein kinase A. Therefore, it is possible that in HEK 293 cells PSD-95 recruits endogenous protein kinases or phosphatases to the receptor complex and induces a change in the phosphorylation state of the receptor. Although phosphorylation sites have not been mapped to specific amino acids located in the NR2A subunit, the C1 exon of the NR1 subunit is known to contain a number of serine residues that have been identified as substrates for phosphorylation by protein kinase A and protein kinase C (Tingley *et al.*, 1997). In addition, the presence of the N1 exon increases the sensitivity of cloned receptors to potentiation by PKC (Kutsuwada *et al.*, 1992; Siegal *et al.*, 1994; Logan *et al.*, 1999). Thus, if PSD-95_{c-Myc} exerts its effects on the radioligand binding properties of the NMDA receptor by altering the phosphorylation state of the receptor, the residues present in the C1 cassette of NR1-1 splice variant may mediate these effects. Furthermore, the effect of PSD-95 may be dependent upon the N1 exon. The effect of switching the NR1-1a splice variant with either the NR1-2a or the NR1-4b splice variants on the PSD-95_{c-Myc}-mediated decrease in the L-glutamate sensitivity of NR1/NR2A receptors was therefore studied.

HEK 293 cells were transfected with either NR1-1a/NR2A $\pm$ PSD-95_{c-Myc}, NR1-2a/NR2A $\pm$ PSD-95_{c-Myc} or NR1-4b/NR2A $\pm$ PSD-95_{c-Myc}. The resulting cell homogenates were assayed for the modulation of [³H]MK801 binding (2 nM) by L-glutamate as described in section 3.8. The enhancement ratios (summarised in Figure 3.32) for the modulation of [³H]MK801 binding by L-glutamate to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} were 1.46 ± 0.16 (n = 3) and 0.98 ± 0.02 (n = 3) respectively. The corresponding ratios for the enhancement of [³H]MK801 binding to NR1-2a/NR2A and NR1-4b/NR2A receptors were 1.35 ± 0.13 (n = 3) and 1.32 ± 0.13 (n = 3) in the presence of PSD-95_{c-Myc} respectively and 0.95 ± 0.05 (n = 3) and 0.99 ± 0.04 (n = 3) in the absence of PSD-95_{c-Myc} respectively. PSD-95_{c-Myc} therefore significantly

($n = 3$; $p < 0.05$, unpaired t test for each receptor subtype) decreased the sensitivity of all three receptor subtypes to L-glutamate. The value of 1.46 ± 0.16 ($n = 3$) obtained for the enhancement of [^3H]MK801 binding to NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc} was slightly below the value of 1.57 ± 0.21 ($n = 11$) obtained in section 3.17. To test whether the magnitude of the PSD-95_{c-Myc}-mediated decrease in the sensitivity to L-glutamate was statistically different between the three different NR1 splice variants, the ratio of 1.46 ± 0.16 was therefore compared with the ratios obtained with the NR1-2a and NR1-4b splice variants. These statistical tests revealed that PSD-95-mediated decrease in the sensitivity to L-glutamate was not dependent on the splice form of the NR1 subunit.

3.22 IMMUNOPRECIPITATION OF NMDA RECEPTOR/PSD-95 COMPLEXES

Having characterised the effects of PSD-95_{c-Myc} on the expression level of NMDA receptors in HEK 293 cells and on their radioligand binding properties, a number of important issues remained unresolved. Firstly, the results of the analysis of [^3H]MK801 binding to NR1-1a/NR2A^{Trunc} receptors in the presence and the absence of PSD-95_{c-Myc} suggested unexpectedly that the NR2A^{Trunc} subunit was able to interact with PSD-95_{c-Myc} although perhaps to a lesser extent. Secondly, the reduced effects of PSD-95_{c-Myc} (C3S/C5S), compared to PSD-95_{c-Myc}, on the expression level of NMDA receptors and on their sensitivity to L-glutamate may not be a direct consequence of the deficient clustering displayed by PSD-95_{c-Myc} (C3S/C5S). Alternatively, the intermediate effects of PSD-95_{c-Myc} (C3S/C5S) compared to wild-type PSD-95_{c-Myc} may in fact be due to a reduced association of PSD-95_{c-Myc} (C3S/C5S) with the NR2 subunit. The immunoblot analysis shown in Figure 3.25 demonstrated a reduced distribution of PSD-95_{c-Myc} (C3S/C5S) to the membrane fraction of transfected HEK 293 cells which may result in a reduced association of PSD-95_{c-Myc} (C3S/C5S) with the NR2A subunit. These two issues were addressed by the immunoprecipitation of NMDA receptor subunits from transfected HEK 293 cells. In this way, the co-association of the NR2A^{WT} and NR2A^{Trunc} subunits with the wild-type and mutant forms of PSD-95_{c-Myc} was investigated.

3.22.1 Quantitative analysis of the soluble and insoluble fractions of lysed HEK 293 cells expressing NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}

A preliminary investigation was performed to determine whether NR1-1a, NR2A and PSD-95_{c-Myc} proteins could be efficiently detergent solubilized from HEK 293 cells expressing NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. HEK 293 cells were transfected with NR1-1a/NR2A receptor clones in the presence and absence of PSD-95_{c-Myc} and cell homogenates were solubilized as described in 2.2.29. Samples of the soluble and insoluble fractions were analysed by quantitative immunoblotting. As shown in Figure 3.33, NR1-1a, NR2A and PSD-95_{c-Myc} were efficiently solubilized in Tris buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 5 mM EDTA and 5 mM EGTA) supplemented with 1 % (v/v) Triton X-100 and 120 mM NaCl. However, the relative solubility of the NR2A subunit was decreased in the presence of PSD-95_{c-Myc}. In the absence of PSD-95_{c-Myc}, 49 ± 3 % ($n = 2$, $\pm$ range) of the total NR2A was solubilized compared to 20 ± 5 % ($n = 2$, $\pm$ range) solubilized in the presence of PSD-95_{c-Myc}. The NR2A subunit was therefore significantly ($n = 2$, $p < 0.05$, unpaired t test) less soluble in the presence of PSD-95_{c-Myc}. In contrast the solubility of the NR1-1a subunit was not influenced by the coexpression of PSD-95_{c-Myc}. The percentage of NR1-1a subunit solubilized from cells expressing NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc} was 81 ± 1 % ($n = 2$, $\pm$ range) and 77 ± 2 % ($n = 2$, $\pm$ range) respectively.

3.22.2 Co-immunoprecipitation of NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}

Initial experiments were performed to determine the most appropriate antibody for the immunoprecipitation of NMDA receptor/PSD-95 complexes. A variety of antibodies were tested because although the anti-NR1 (17-35), anti-NR1 (911-920), anti-NR2A (1,381-1,394) antibodies were excellent for detecting their corresponding subunits by immunoblotting, the binding of these antibodies to their epitopes in native protein had not been characterised in the presence of PSD-95. It is possible the interaction of PSD-95 with the intracellular C-terminal region of the NR2 subunit may have interfered with the binding of the anti-NR1 (911-920) and the anti-NR2A (1,381-1,394) antibodies to their respective NMDA receptor subunits because the epitopes are located in close proximity to the site of the interaction between PSD-95 and the NR2 subunit. Immunoprecipitation reactions were therefore carried out as described in 2.2.29 from cells expressing either

NR1-1a/NR2A/PSD-95_{c-Myc} or PSD-95_{c-Myc} alone using the anti-NR1 (17-35), anti-NR1 (911-920), anti-NR2A (1,381-1,394) antibodies and control, non-immune immunoglobulins (IgG). The immunoprecipitation reactions from HEK 293 cells transfected with PSD-95_{c-Myc} alone were performed to verify the specificity of these antibodies for their proposed antigens and to discount cross-reactivity with PSD-95_{c-Myc}. As shown by the analysis of transfected HEK 293 cell homogenates by immunoblotting (Figure 3.30), anti-NR1 (17-35) antibodies unexpectedly labelled PSD-95_{c-Myc}, and may therefore have immunoprecipitated PSD-95_{c-Myc} from HEK 293 cells expressing PSD-95_{c-Myc} alone. The resulting immune pellets were analysed by immunoblotting and assayed for the presence of NR1-1a, NR2A and PSD-95_{c-Myc} immunoreactivity. Representative immunoblots are shown in Figure 3.34. The immune pellets obtained from HEK 293 cells transfected with NR1-1a/NR2A/PSD-95_{c-Myc} with anti-NR1 (17-35) antibodies were immunoreactive for NR1-1a, NR2A and PSD-95_{c-Myc}, suggesting the co-immunoprecipitation of an NR1/NR2A/PSD-95_{c-Myc} complex. None of the NMDA receptor subunits or PSD-95_{c-Myc} were detected in the immune pellets collected with non-immune IgG. However, and again surprisingly, the anti-NR1 (17-35) antibodies displayed cross-reactivity with PSD-95_{c-Myc} since the immune pellets precipitated with this antibody from HEK 293 cells transfected with PSD-95_{c-Myc} alone were shown to contain PSD-95_{c-Myc} immunoreactivity. PSD-95_{c-Myc} was not detected in the immune pellets collected with non-immune IgG. In contrast to anti-NR1 (17-35) antibodies, the anti-NR1 (911-920) antibodies did not display a similar cross-reactivity with PSD-95_{c-Myc} and thus demonstrated specificity for the NR1 subunit. However, PSD-95_{c-Myc} was not detected in the immune pellets obtained from HEK 293 cells transfected with NR1-1a/NR2A/PSD-95_{c-Myc} despite the abundance of NR1-1a immunoreactivity in the immune pellets. Anti-NR1 (911-920) antibodies did not therefore efficiently co-immunoprecipitate NR1-1a/NR2A/PSD-95_{c-Myc} complexes. Lastly, immune pellets obtained with NR2A (1,381-1,394) antibodies were immunoreactive for NR2A, NR1-1a and PSD-95_{c-Myc}. PSD-95_{c-Myc} was not detected in the immune pellets obtained with either control IgG from cells transfected with NR1-1a/NR2A/PSD-95_{c-Myc}, or with NR2A (1,381-1,394) antibodies from cells transfected with PSD-95_{c-Myc} alone. Anti-NR2A (1,381-1,394) antibodies therefore co-immunoprecipitated NR2A/PSD-95_{c-Myc} complexes and were the antibodies of choice to investigate further the possible association of both NR2A^{Trunc} with PSD-95_{c-Myc}, and NR2A^{WT} with PSD-95_{c-Myc} (C3S/C5S).

Quantitative immunoprecipitation of NR1-1a/NR2A receptors from HEK 293 cells transfected in the presence and absence of PSD-95_{c-Myc}

It is interesting to note that the amount of NR2A subunit immunoprecipitated from HEK 293 cells transfected with NR1-1a/NR2A receptors was significantly reduced in the presence of PSD-95_{c-Myc}. Cell homogenates expressing NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} were solubilized and immunoprecipitation reactions were performed using anti-NR2A (1,381-1394) and anti-NR1 (911-920) antibodies. The immune pellets were analysed by quantitative immunoblotting (summarised in Figure 3.35). The amount of NR2A subunit immunoprecipitated with anti-NR2A (1,381-1,394) antibodies from HEK 293 cells expressing NR1-1a/NR2A receptors was decreased by 51 ± 11 % ($n = 6$; $p < 0.0001$, paired t test) in the presence of PSD-95_{c-Myc}. A similar effect on the NR2A subunit was observed by immunoprecipitation with anti-NR1 (911-920) antibodies. The intensity of NR2A immunoreactivities detected in immune pellets obtained with anti-NR1 (911-920) antibodies was decreased by 68 ± 3 % ($n = 2$, $\pm$ range; $p < 0.05$, paired t test) in the presence of PSD-95_{c-Myc}. The likely explanation for this difference is the reduced solubility of the NR2A subunit in the presence of PSD-95_{c-Myc}.

The level of NR1-1a immunoreactivity detected in immune pellets precipitated with anti-NR1 antibodies was not significantly different between cells expressing NR1-1a/NR2a receptors either in the presence or the absence of PSD-95_{c-Myc}. This agrees with the analysis performed in section 3.22.1, which revealed that the solubility of NR1-1a was unchanged by the co-expression of PSD-95_{c-Myc}. However, the level of NR1 immunoreactivity detected in immune pellets precipitated with anti-NR2A (1,381-1,394) antibodies was decreased by 80 ± 5 % ($n = 2$, $\pm$ range; $p < 0.05$, paired t test) in the presence of PSD-95_{c-Myc}. Since PSD-95_{c-Myc} reduces the amount of NR2A immunoprecipitated from transfected HEK 293 cells, it follows that the amount of NR1 co-immunoprecipitated with NR2A in the presence of PSD-95_{c-Myc} would also be reduced.

3.22.3 Does PSD-95_{c-Myc} co-associate with NR1-1a/NR2A^{Trunc} receptors expressed in HEK 293 cells?

HEK 293 cells were transfected with NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptor clones both in the presence of PSD-95_{c-Myc}. The resulting cell homogenates were detergent solubilized and immunoprecipitation reactions were performed using anti-NR2A (1,381-1,394) and non-immune IgG as control. Immune pellets were analysed by

immunoblotting using anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as shown in Figure 3.36. As expected the immune pellets obtained from cells transfected with NR1-1a/NR2A^{WT}/PSD-95_{c-Myc} by immunoprecipitation with anti-NR2A antibodies were immunoreactive for NR2A^{WT} and PSD-95_{c-Myc}. The immune pellets collected from cells transfected with NR1-1a/NR2A^{Trunc}/PSD-95_{c-Myc} contained NR2A^{Trunc} subunit and also PSD-95_{c-Myc} (Figure 3.36, lane 6). PSD-95_{c-Myc} was not detected in the immune pellets obtained with control IgG demonstrating that indeed the NR2A^{Trunc} subunit does interact with PSD-95_{c-Myc}. The amount of PSD-95_{c-Myc} co-immunoprecipitated with NR2A^{Trunc} was decreased by $51 \pm 16 \%$ ($n = 3$) compared to the amount co-immunoprecipitated with NR2A^{WT} suggesting that the NR2A^{Trunc} subunit does associate with PSD-95_{c-Myc} but with a reduced affinity compared to NR2A^{WT}.

3.22.4 Does PSD-95_{c-Myc}(C35/S35) co-associate with NR1-1a/NR2A receptors expressed in HEK 293 cells?

NR2A^{WT} subunits were immunoprecipitated from detergent solubilized HEK 293 cells expressing NR1-1a/NR2A^{WT} receptors in the presence of either PSD-95_{c-Myc} or PSD-95_{c-Myc}(C35/S35) using anti-NR2A (1,381-1,394) antibodies. Immune pellets were analysed by immunoblotting using anti-NR2A (1,381-1,394) and anti-c-Myc antibodies (Figure 3.36). The analysis revealed that both PSD-95_{c-Myc} and PSD-95_{c-Myc}(C35/S35) were co-immunoprecipitated with the NR2A^{WT} subunit. Furthermore, the amount of PSD-95_{c-Myc}(C35/S35) detected in the immune pellets was comparable to that of PSD-95_{c-Myc}. Therefore, the mutant PSD-95_{c-Myc} does associate with NR2A^{WT} and apparently to the same extent as the wild-type PSD-95_{c-Myc}.

3.23 INVESTIGATION OF THE EFFECT OF PSD-95_{c-Myc} ON THE PHOSPHORYLATION NR1 AND NR2 SUBUNITS

The potential role of phosphorylation in the PSD-95_{c-Myc}-induced decrease in the sensitivity of NR1/NR2A receptors to L-glutamate was investigated in section 3.21. Even though the effect of PSD-95_{c-Myc} on the sensitivity of [³H]MK801 binding to L-glutamate was not dependent upon the N1, C1 or C2 exons, phosphorylation sites in other regions of the NR1 subunit and in the NR2A subunit may mediate the effects of PSD-95. The strategy utilised to test this hypothesis was to analyse NR1-1a and NR2A subunits

immunoprecipitated from solubilized HEK 293 cells expressing NR1-1a/NR2A $\pm$ PSD-95_{c-Myc} by quantitative immunoblotting using anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies. A comparative analysis of the intensities of their respective immunoreactivities in the presence and absence of PSD-95_{c-Myc} may detect a PSD-95_{c-Myc}-induced change in the phosphorylation state of either the NR1-1a or NR2A subunit. Since the amount of NR2A subunit immunoprecipitated from transfected HEK 293 cell lysates was reduced in the presence of PSD-95_{c-Myc}, the intensities of the resulting anti-phospho-(S/T/Y) antibody immunoreactivities would have to be normalized to the intensity of the immunoreactivities labelled with the anti-NR2A (1,381-1,394) and anti-NR1 (911-920) antibodies.

HEK 293 cells were transfected with NR1-1a/NR2A receptor clones in the presence and absence of PSD-95_{c-Myc} and immunoprecipitation reactions were carried out using anti-NR2A (1,381-1,394) and anti-NR1 (911-920) antibodies. Immune pellets were analysed by quantitative immunoblotting using the anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies as described in 2.2.29. Nitrocellulose membranes were then re-probed using anti-NR2A (1,381- 1,394) and anti-NR1 (911-920) antibodies to control for any difference in the level of NR2A or NR1-1a subunit immunoreactivities. The anti-phosphoserine and anti-phosphothreonine antibodies labelled NR1-1a and NR2A subunits with very weak intensities and with high levels of background immunoreactivity both in the presence and absence of PSD-95_{c-Myc}. This indicated that the NR1-1a and NR2A subunits either exhibited very low levels of serine/threonine phosphorylation in HEK 293 cells or the antibodies did not recognise phosphorylated serine or threonine residues. As shown in Figure 3.37, the anti-phosphotyrosine antibody did not label the NR2A subunit immunoprecipitated from HEK 293 cells transfected either in the presence or the absence of PSD-95_{c-Myc}. However, the NR1-1a subunit was labelled with greater intensity using the anti-phosphotyrosine antibody. To determine whether the anti-phosphotyrosine antibody specifically labelled phosphorylated tyrosine residues, the antibody was pre-incubated with phosphotyrosine as described in section 2.2.29. The results of this analysis are shown in Figure 3.38. Pre-incubation with phosphotyrosine did not prevent the labelling of NR1-1a with the anti-phosphotyrosine antibody, demonstrating that this antibody did not specifically label phosphorylated NR1 subunit tyrosine residues. Further investigations into the phosphorylation state of NMDA receptors subunits using these antibodies were therefore not performed.

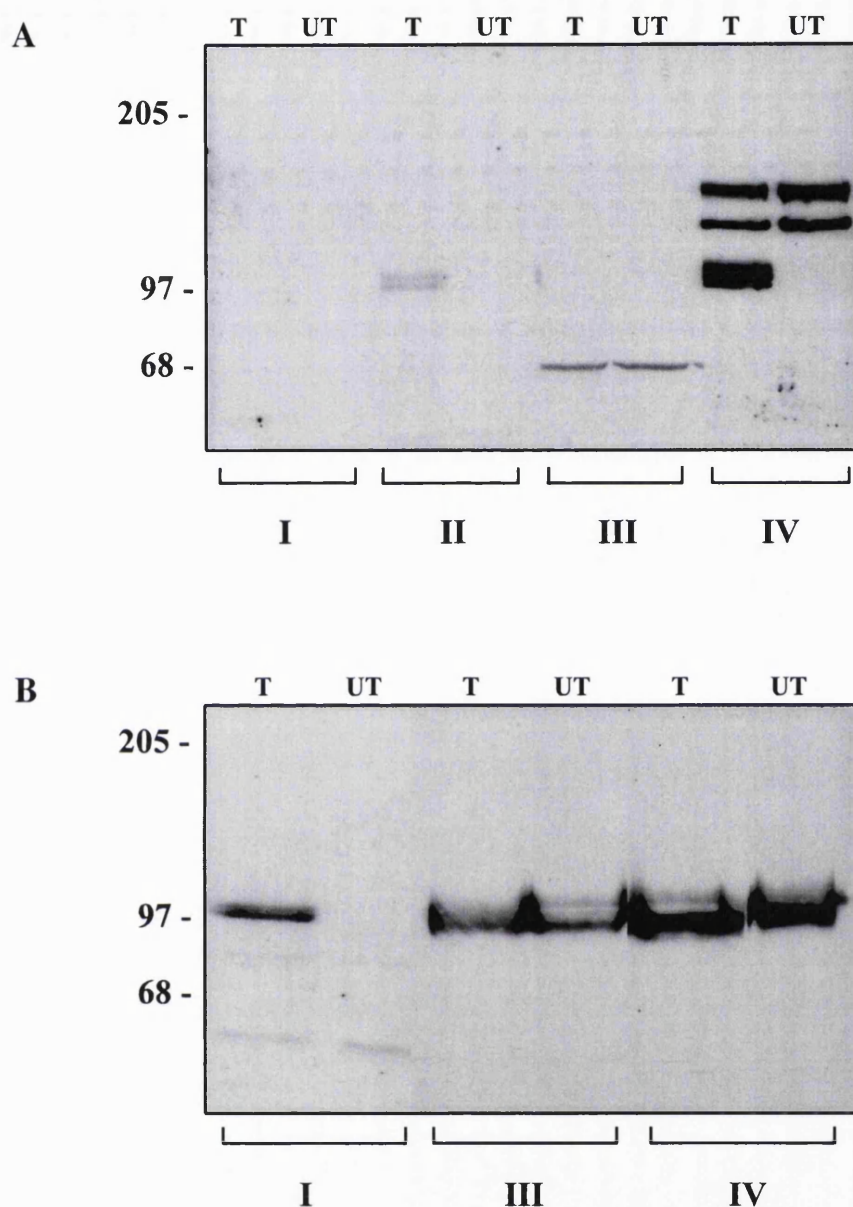


Figure 3.1.1 Immunoblots demonstrating the transient expression of PSD-95 in HEK 293 cells and the characterisation of anti-PSD-95 (480-498) antibodies

HEK 293 cells were transfected with pGW1PSD-95 as described in 2.2.21. Cell homogenates were prepared (2.2.24) from transfected (T) and untransfected cells (UT). Immunoblotting was carried out as described in 2.2.28 using the following antibodies. **A.** Anti-PSD-95 (480-498) antibodies from rabbit I, bleeds I-IV as indicated. **B.** Anti-PSD-95 (480-498) antibodies from rabbit II, bleeds I, III, and IV as indicated. The positions of the molecular weight standards are shown on the left.

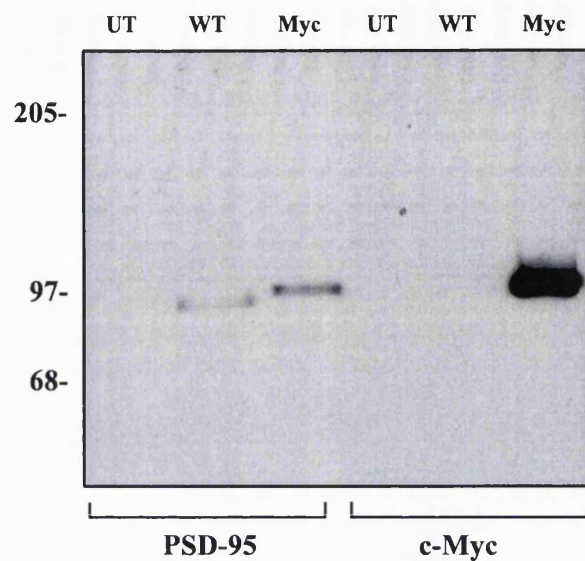


Figure 3.1.2 Immunoblot demonstrating the transient expression of PSD-95 and PSD-95_{c-Myc} in HEK 293 cells

HEK 293 cells were transfected with either pGW1PSD-95 (WT) or pGW1PSD-95_{c-Myc} (Myc) as described in 2.2.21. Cell homogenates were prepared (2.2.24) from transfected and untransfected (UT) cells, and analysed by immunoblotting using anti-c-Myc 9E10 mouse monoclonal and anti-PSD-95 (480-498) antibodies from rabbit I, bleed II as indicated. The positions of the molecular weight standards are shown on the left.

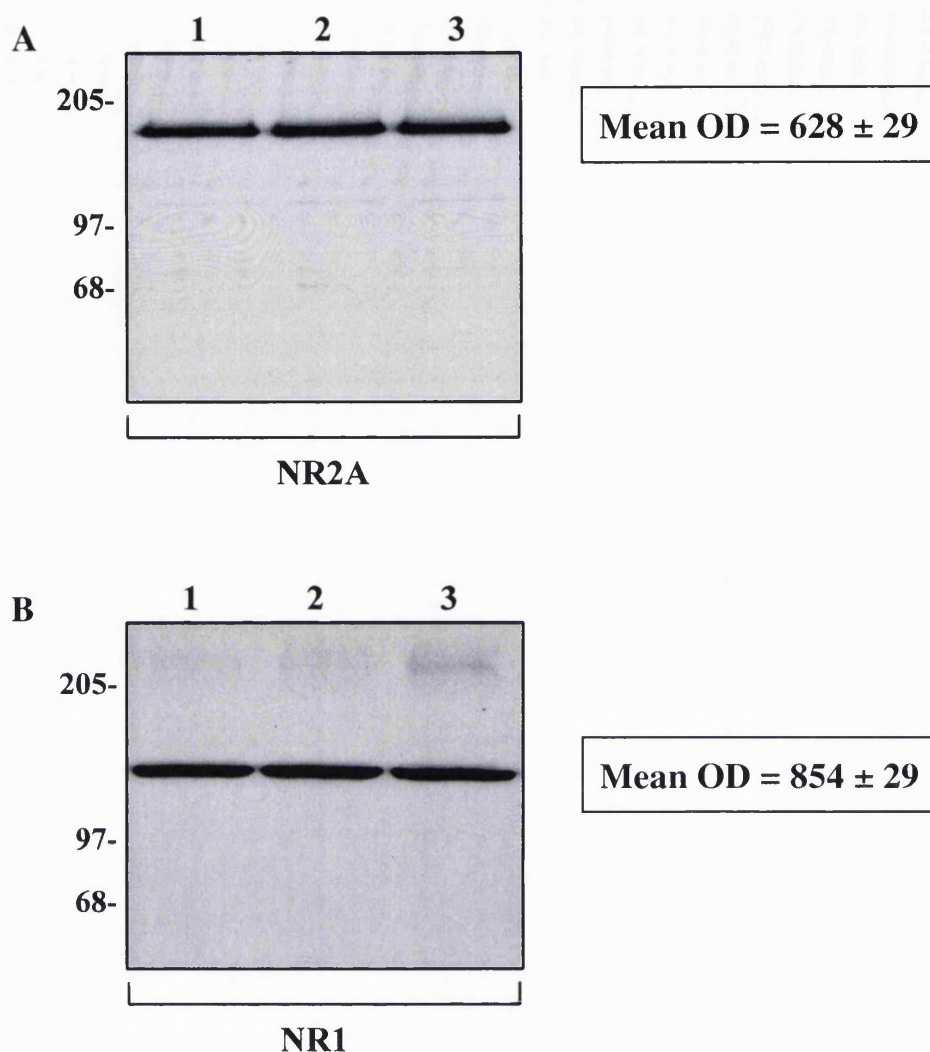


Figure 3.2 Immunoblots demonstrating the reproducibility of quantitative immunoblotting

HEK 293 cells were transfected with either pCISNR1-1a or pCISNR2A receptor clones. The resulting cell homogenates were analysed by quantitative immunoblotting. **A.** Triplicate samples (lanes 1-3) of cell homogenate from a single flask of HEK 293 cells transfected with pCISNR2A immunoblotted using the anti-NR2A (1,381-1,394) antibodies. The mean $\pm$ standard deviation (SD) intensity of this single triplicate analysis is shown on the right. **B.** Single samples of cell homogenate from three flasks of HEK 293 cells (lanes 1-3) separately transfected with pCISNR1-1a immunoblotted using the anti-NR1 (911-920) antibodies. The mean $\pm$ SD intensity of these single immunoreactive bands from $n = 3$ separate transfections is shown on the right. In **A** and **B** the positions of molecular weight standards are shown on the left.

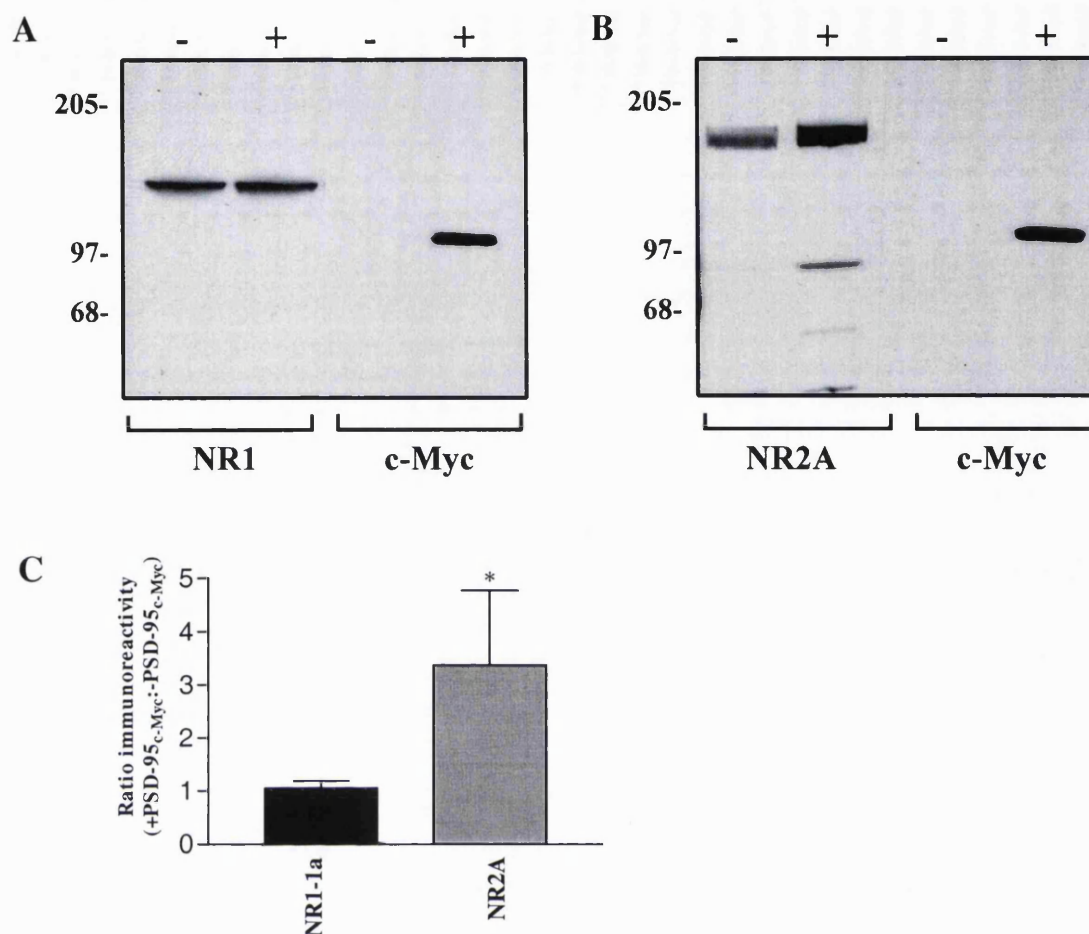


Figure 3.3 The effect of the co-expression of PSD-95_{c-Myc} in HEK 293 cells on the expression level of single NR1-1a and NR2A subunits

HEK 293 cells were transfected with either pCISNR1-1a or pCISNR2A, both in the presence (+) and absence (-) of pGW1PSD-95_{c-Myc}. The resulting cell homogenates were analysed by quantitative immunoblotting using the following antibodies. **A.** HEK 293 cells transfected with NR1-1a in the presence (+) and absence (-) of PSD-95_{c-Myc} using the anti-NR1 (911-920) and anti-c-Myc antibodies as indicated. **B.** HEK 293 cells transfected with NR2A in the presence (+) and absence (-) of PSD-95_{c-Myc} using the anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. The positions of molecular weight standards are shown on the left. **C.** Histogram summarising the results of the quantitative immunoblotting. Results are expressed as the ratio of the immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to the intensities in the absence of PSD-95_{c-Myc}. Ratios are means $\pm$ SD (bars) of the analyses from at least $n = 3$ independent transfections with $n = 3$ immunoblots per transfection. Fold changes in immunoreactive intensities were analysed by paired students t tests ($*p < 0.05$).

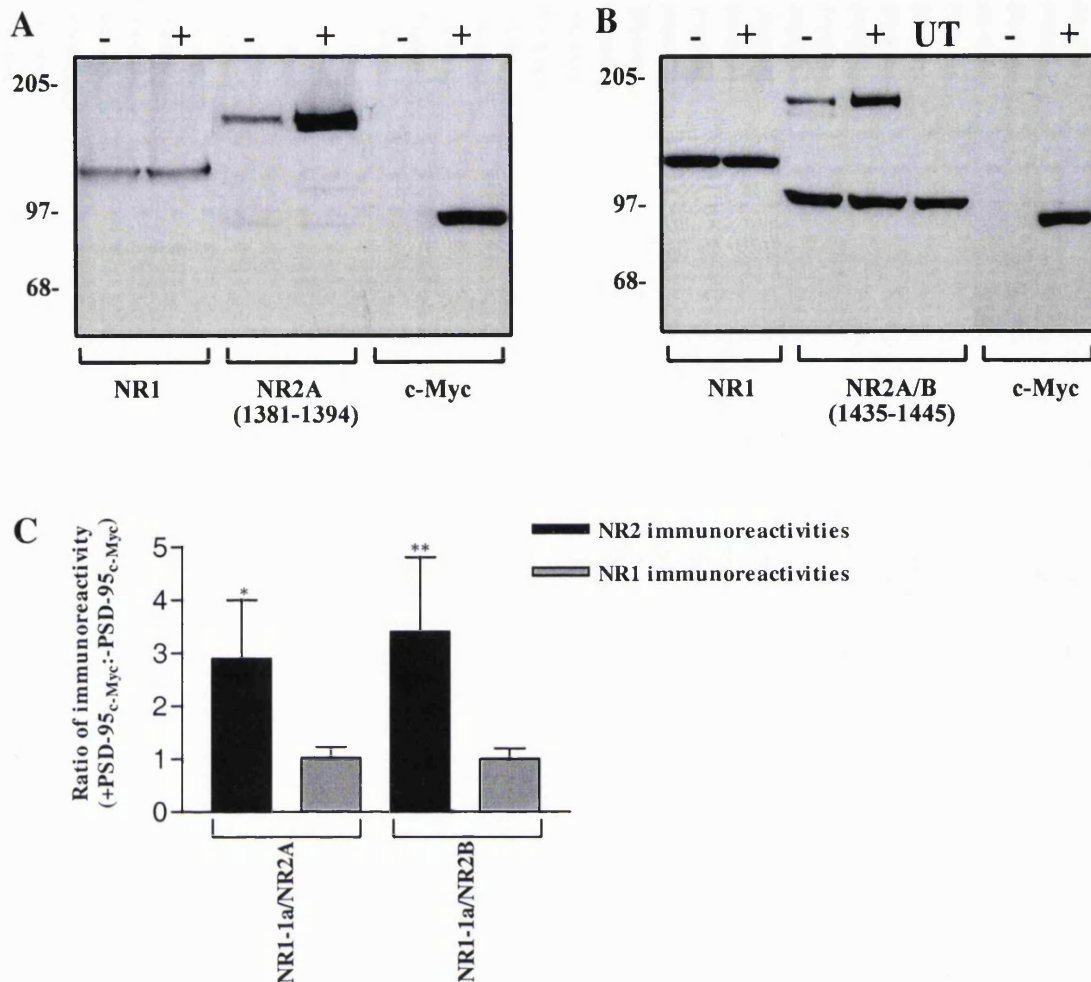


Figure 3.4 Immunoblots demonstrating the effect of PSD-95_{c-Myc} on the level of NR1-1a/NR2A and NR1-1a/NR2B receptors expressed in HEK 293 cells

HEK 293 cells were transfected with either NR1-1a/NR2A or NR1-1a/NR2B in the presence and absence of PSD-95_{c-Myc}. The resulting cell homogenates were analysed by quantitative immunoblotting. **A.** HEK 293 cells transfected with NR1-1a/NR2A in the presence (+) and absence (-) of PSD-95_{c-Myc} immunoblotted with anti-NR1 (911-20), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. **B.** HEK 293 cells transfected with NR1-1a/NR2B receptors in the presence (+) and absence (-) of PSD-95_{c-Myc} and untransfected cells (UT) immunoblotted with anti-NR1 (911-920), anti-NR2A/B (1,435-1,445) and anti-c-Myc antibodies as indicated. The positions of the molecular weight markers are shown on the left. **C.** Histogram summarising the results of the quantitative immunoblotting. Results are expressed as the ratio of the immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to the intensities measured in the absence of PSD-95_{c-Myc} and are means $\pm$ SD (bars) of at least $n = 3$ separate transfections with $n = 3$ immunoblots per transfection. Fold changes in immunoreactive intensities were analysed by a paired students t tests (* $p < 0.001$, ** $p < 0.01$).

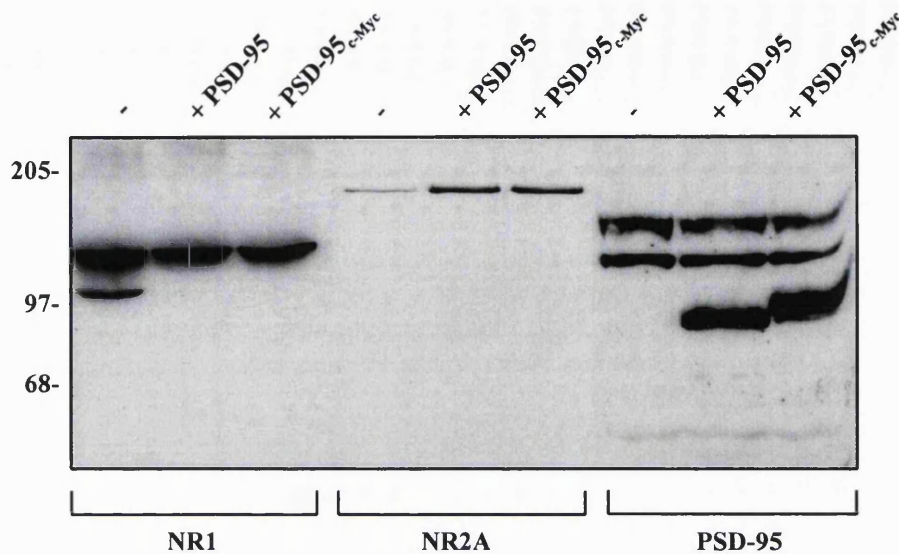


Figure 3.5 A comparison of the effects of PSD-95 and PSD-95_{c-Myc} on the expression of NR1-1a/NR2A receptors in HEK 293 cells

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of either wild type PSD-95 or PSD-95_{c-Myc}. The resulting cell homogenates were analysed by immunoblotting using anti-NR1 (911-920), anti-NR2A (1,381-1,394) and anti-PSD-95 (480-498; rabbit I, bleed IV) as indicated. The positions of the molecular weight standards are shown on the left. The immunoblots shown are from a single experiment.

Transfection conditions	Percentage cell death	
PSD-95 _{c-Myc}	0	(1)
NR1-1a/NR2A	22 ± 3	(3)
NR1-1a/NR2A/PSD-95 _{c-Myc}	20	(1)
NR1-1a/NR2B	25 ± 3	(2)
NR1-1a/NR2B/PSD-95 _{c-Myc}	26 ± 2	(2)

Table 3.1 The effect of PSD-95_{c-Myc} on NMDA receptor-mediated cell cytotoxicity

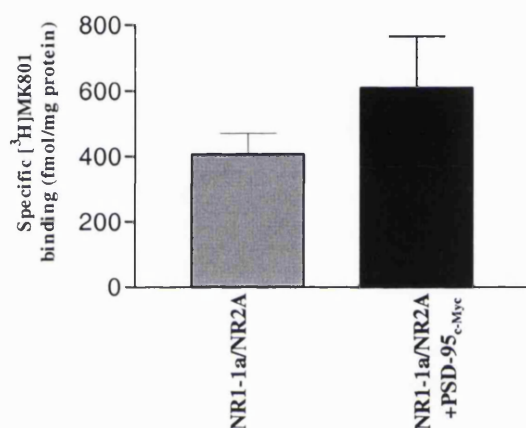
HEK 293 cells were transfected with PSD-95_{c-Myc} alone and with either NR1-1a/NR2A or NR1-1a/NR2B in the presence and absence of PSD-95_{c-Myc}. Cells were cultured post-transfection in the presence and absence of 1 mM ketamine for 16 h. Percentage cell death was determined using the CytotoxTM kit as described in 2.2.23. Triplicate values were determined per transfection and the results are the means ± SD of n = 3 replicate transfections or the means ± range of n = 2 replicate transfections.

A

Receptor preparation	Single point [³ H]MK801 binding (fmol/mg protein)		Single point [³ H]MDL 105,519 binding (fmol/mg protein)	
NR1-1a/NR2A	410 ± 60	(5)	1.6 ± 0.1	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	610 ± 160*	(6)	1.5 ± 0.1	(6)

**P* < 0.01

B



C

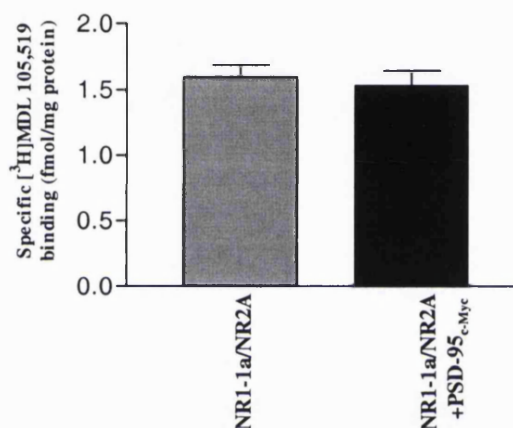


Figure 3.6 Single point [³H]MK801 and [³H]MDL 105,519 radioligand binding to HEK 293 cells transfected with NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were prepared (2.2.24) and analysed by single point [³H]MK801 (25 nM) and [³H]MDL 105,519 (1 nM) radioligand binding as described in 2.2.30.2 and 2.2.30.3, with 100 μM TCP and 100 μM glycine for the determination of non-specific binding respectively. The results are shown in **A**. Values are means ± SD of triplicate determinations from (n) independent transfections. The effects of PSD-95_{c-Myc} were analysed using unpaired students *t* tests. The levels of significance are shown below the table. Total [³H]MK801 binding in the presence and absence of PSD-95_{c-Myc} was in the range 2400-2900 and 2100-2300 dpm respectively, with non-specific binding of 1000-1300 dpm in both transfection conditions. Total [³H]MDL 105,519 binding was in the range 16,000-20,000 dpm in both transfection conditions, with non-specific binding of 1,800-2,500 dpm. **B** and **C**. Histograms summarising the specific binding of [³H]MK801 (**B**) and [³H]MDL 105,519 (**C**) to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}.

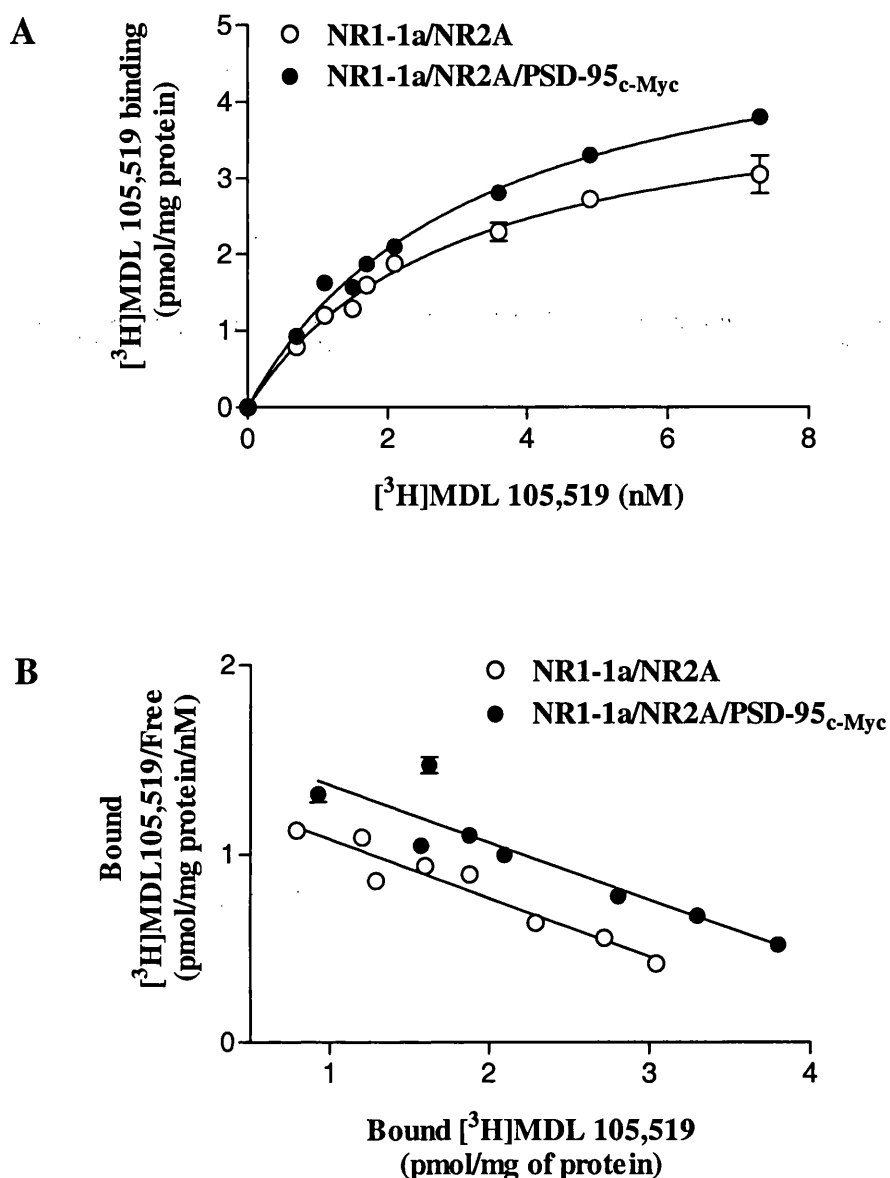


Figure 3.7 Saturation analysis and Rosenthal transformation of ^3H MDL 105,519 radioligand binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were prepared and assayed for ^3H MDL 105,519 radioligand binding as described in 2.2.30.3, with 100 μM glycine for the determination of non-specific binding. **A** and **B**. Representative saturation curve (**A**) and Rosenthal transformation (**B**) for the specific binding of ^3H MDL 105,519 to NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Analysis of the curves by non-linear regression resolved single, high affinity binding sites in the presence and absence of PSD-95_{c-Myc} with binding parameters summarised in Table 3.2. Data points are means $\pm$ SD (bars) of triplicate determinations from an experiment repeated at least twice.

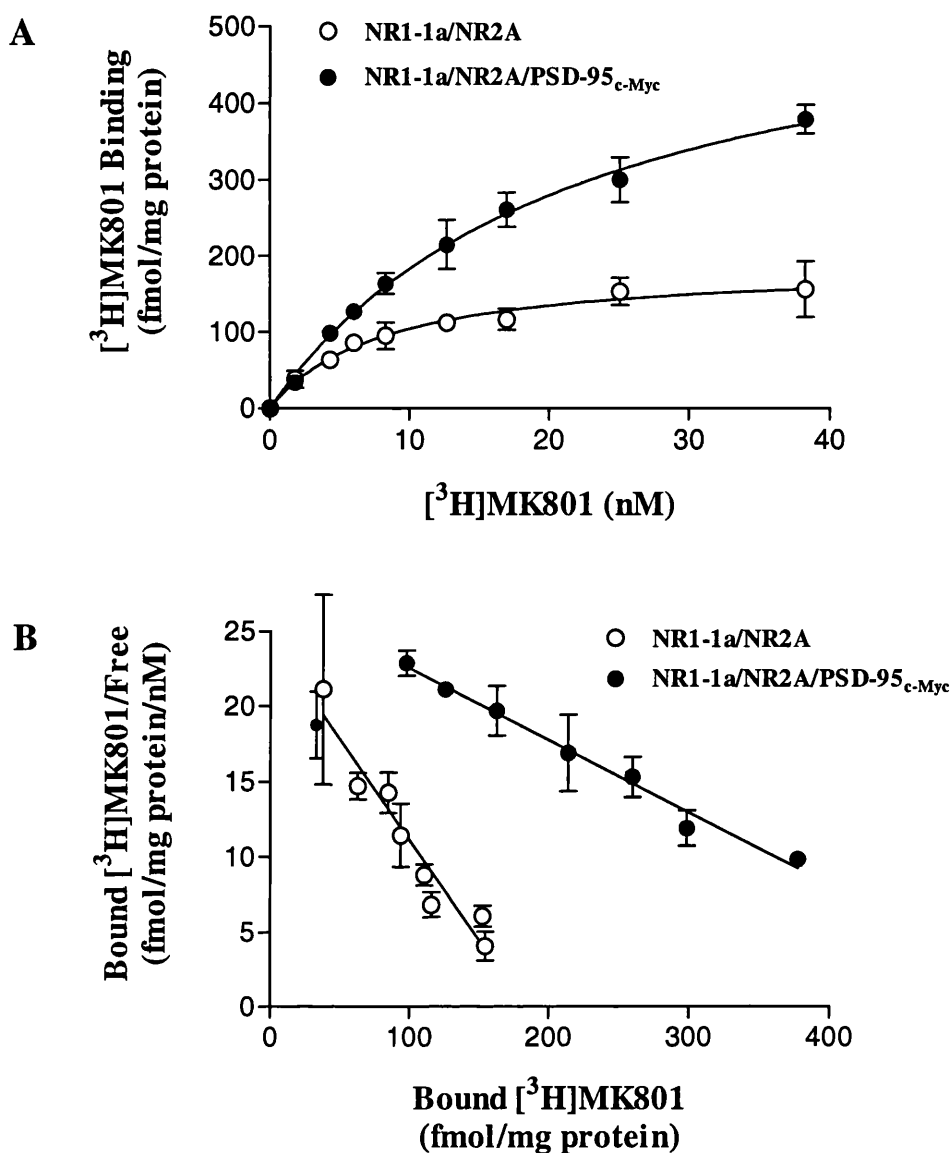


Figure 3.8 Saturation analysis and Rosenthal transformation of $^{[3]H}$ MK801 radioligand binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were prepared and assayed for $^{[3]H}$ MK801 radioligand binding in the presence of 30 μ M glycine and 10 μ M L-glutamate as described in 2.2.30.2, with 100 μ M TCP for the determination of non-specific binding. **A** and **B**. Representative saturation curve (**A**) and Rosenthal transformation (**B**) for the specific binding of $^{[3]H}$ MK801 to NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Analysis of the curves by non-linear regression resolved single, high affinity binding sites in the presence and absence of PSD-95_{c-Myc} with binding parameters summarised in Table 3.2. Data points are means $\pm$ SD (bars) of triplicate determinations from an experiment repeated at least three times.

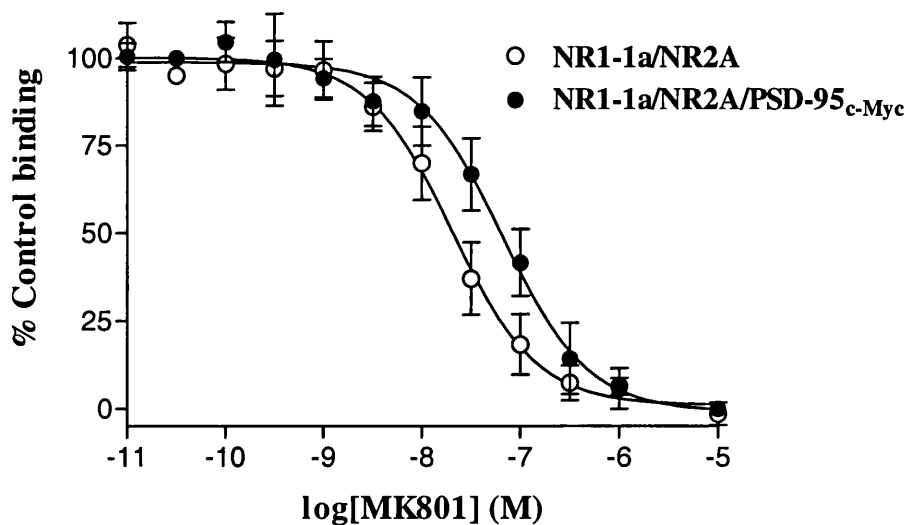


Figure 3.9 Inhibition curves for the displacement of [³H]MK801 by MK801 from NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cell homogenates were assayed for [³H]MK801 radioligand binding (10 nM) in the presence of a 0 - 10 μM range of MK801 concentrations, 30 μM glycine and 10 μM L-glutamate as described in 2.2.30.2, with 100 μM TCP for the determination of non-specific binding. Results are expressed as a percentage of control [³H]MK801 specific binding, *i.e.* specific [³H]MK801 binding assayed in the absence of added MK801. Data points are means ± SD (bars) of the combined determinations from *n* = 5 independent transfections. Analysis of the curves by non-linear regression (GraphPad Prism) resolved single binding sites both in the presence and absence of PSD-95_{c-Myc}, with *K_i* values and Hill coefficients summarised in Table 3.2. Typical means ± SD of the total binding determined in triplicate in the absence of added MK801 from a single transfection were 2990 ± 70 and 2340 ± 60 dpm in the presence and absence of PSD-95_{c-Myc} respectively, with corresponding non-specific binding determinations of 840 ± 10 and 950 ± 70 dpm.

A. [³H]MDL 105,519 binding parameters

Receptor preparation	K _D (nM)		B _{max} (pmol/mg protein)	
NR1-1a/NR2A	3.5 ± 0.1	(2)	4.2 ± 0.1	(2)
NR1-1a/NR2A/PSD-95 _{c-Myc}	3.3 ± 0.1	(2)	4.2 ± 1.2	(2)

B. [³H]MK801 binding parameters

Receptor preparation	K _D (nM)		B _{max} (fmol/mg protein)		K _I (nM)		Hill coefficient	
NR1-1a/NR2A	14 ± 4	(5)	330 ± 130	(5)	12 ± 7	(5)	1.06 ± 0.15	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	37 ± 18*	(5)	920 ± 480 **	(5)	59 ± 19***	(5)	1.08 ± 0.13	(5)
Rat forebrain P2	ND		ND		13 ± 8	(3)	0.92 ± 0.02	(3)

p*<0.05; *p*<0.05, paired *t* test; ****p*<0.005

Table 3.2 Summary and comparison of the parameters for [³H]MK801 and [³H]MDL 105,519 radioligand binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

Binding parameters were determined for [³H]MDL 105,519 (A) and [³H]MK801 (B) radioligand binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} and to rat forebrain membranes. Binding curves were analysed using GraphPAD Prism software. Values are means ± SD of at least n = 3 determinations or means ± range of n = 2 (n number shown in brackets). ‘ND’ is not determined. The statistical analysis of the effects of PSD-95_{c-Myc} on the radioligand binding parameters of NR1-1a/NR2A receptors was performed using unpaired students *t* tests unless otherwise stated. Levels of significance are indicated by an asterisk. The statistical analysis of the effect of PSD-95_{c-Myc} on the B_{max} value for [³H]MK801 binding to NR1-1a/NR2A receptors was performed using a paired students *t* test to allow for variations in transfection efficiency.

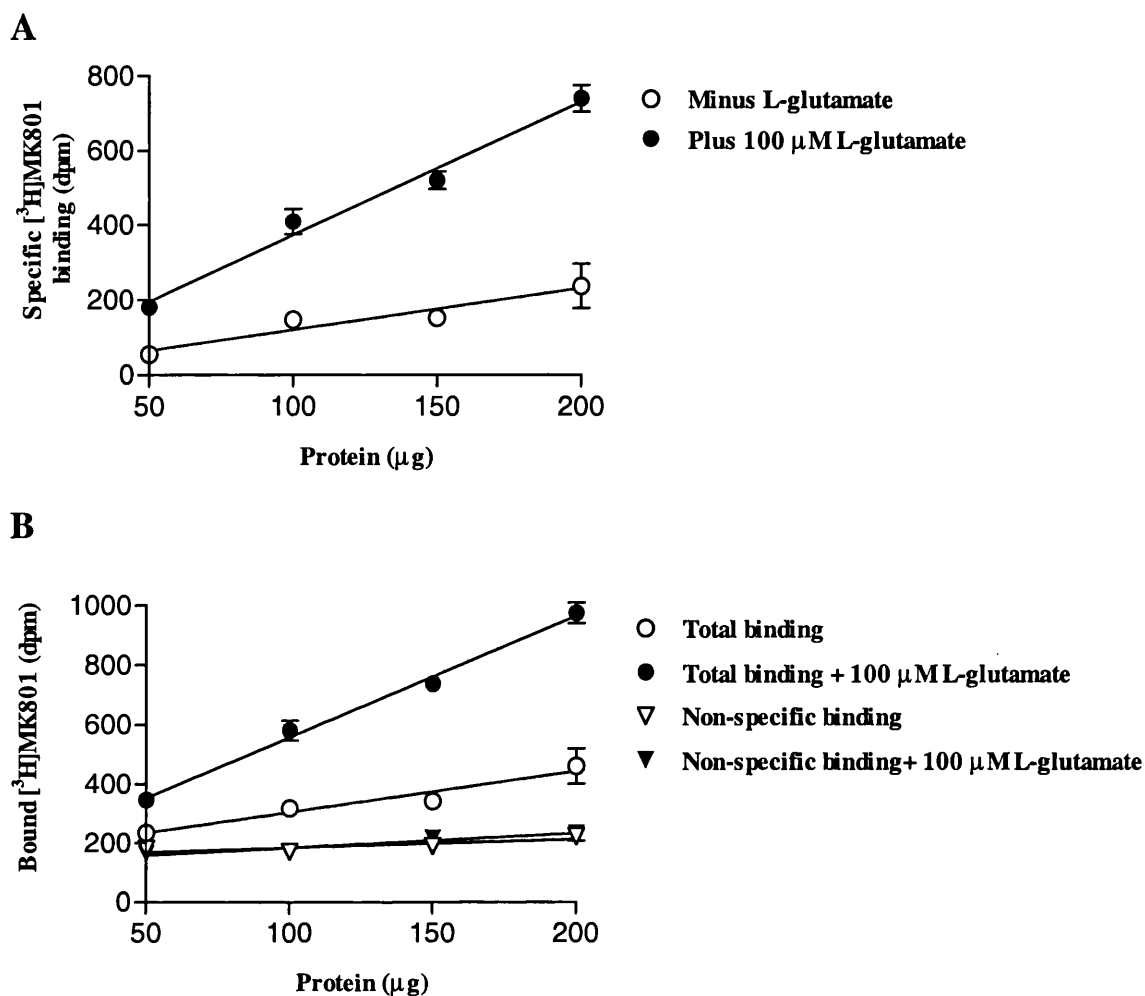


Figure 3.10 Optimization of the [^3H]MK801 radioligand binding assay to study the allosteric modulation of [^3H]MK801 binding to NR1-1a/NR2A receptors by L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A receptors. Cells were harvested and washed extensively as described in 2.2.30.2. A range of protein concentrations (50-200 μg per assay tube) were assayed for [^3H]MK801 binding (2 nM) in the presence and the absence of 100 μM L-glutamate, using 100 μM TCP for the determination of non-specific binding. **A.** Specific [^3H]MK801 (2 nM) binding to NR1-1a/NR2A receptors in the presence (closed symbols) and absence (open symbols) of 100 μM L-glutamate. **B.** Total and non-specific [^3H]MK801 (2 nM) binding in the presence (closed symbols) and absence (open symbols) of 100 μM L-glutamate. Data points are means $\pm$ SD (bars) of triplicate determinations from a single transfection. The enhancement of specific and non-specific [^3H]MK801 binding by L-glutamate at each protein concentration is summarised in Table 3.3.

Protein concentration (μg per assay tube)	Enhancement of specific binding (%) by L-glutamate	Enhancement of non-specific binding (%) by L-glutamate	Non-specific binding (% of total) minus L-glutamate	Non-specific binding (% of total) plus L-glutamate
50	232 $\pm$ 31	-9 $\pm$ 10	77 $\pm$ 5	48 $\pm$ 5
100	177 $\pm$ 19	0 $\pm$ 4	53 $\pm$ 2	29 $\pm$ 1
150	240 $\pm$ 13	14 $\pm$ 7	55 $\pm$ 3	30 $\pm$ 2
200	212 $\pm$ 15	5 $\pm$ 10	48 $\pm$ 5	24 $\pm$ 2

Table 3.3 Optimal conditions for the enhancement of [^3H]MK801 binding to NR1-1a/NR2A receptors by L-glutamate and for the reduction of non-specific [^3H]MK801 binding

The table shows the percentage enhancement of specific and non-specific [^3H]MK801 (2 nM) binding to NR1-1a/NR2A receptors by 100 μM L-glutamate. A range of protein concentrations were assayed (50 - 200 μg protein per assay tube). The level of non-specific [^3H]MK801 (2 nM) binding in the presence and absence of 100 μM L-glutamate at each protein concentration is expressed as a percentage of the total [^3H]MK801 binding, *i.e.* specific plus non-specific binding. The results are the means $\pm$ SD of triplicate determinations from a single transfection.

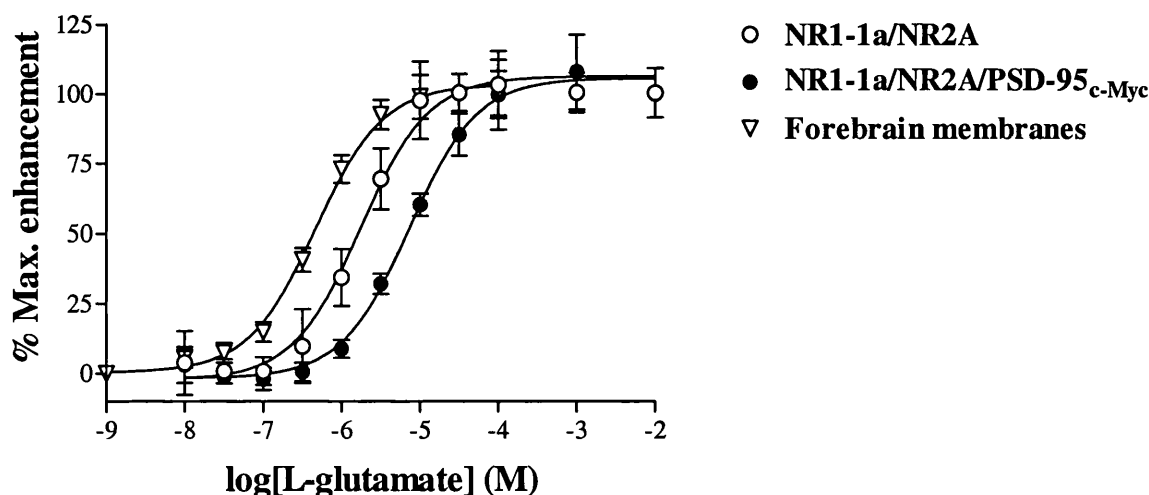


Figure 3.11 Enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} and rat forebrain membranes by L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95. Cells were harvested and washed extensively. The resulting cell homogenates and rat forebrain membranes (▽) were assayed for [³H]MK801 binding (2 nM) in the presence of 30 μM glycine and in a range of L-glutamate concentrations (0 - 10 mM). The enhancement of [³H]MK801 specific binding by L-glutamate at each point was calculated as the percentage of the control specific binding, *i.e.* specific [³H]MK801 binding measured in the absence of added L-glutamate. It is expressed as a percentage of the maximum enhancement induced by 10 mM L-glutamate. Data points are means ± SD (bars) of the combined determinations from at least n = 3 independent transfections. EC₅₀ values, percentage maximum enhancement determinations and Hill coefficients are summarised in Table 3.4. Typical increases in triplicate determinations for specific [³H]MK801 (2 nM) binding to NR1-1a/NR2A receptors induced by the addition of 1 mM L-glutamate compared to control binding, *i.e.* in the absence of added L-glutamate, were 610 ± 10 to 2500 ± 40 dpm in the presence of PSD-95_{c-Myc}, and 270 ± 30 to 940 ± 50 dpm in the absence of PSD-95_{c-Myc}.

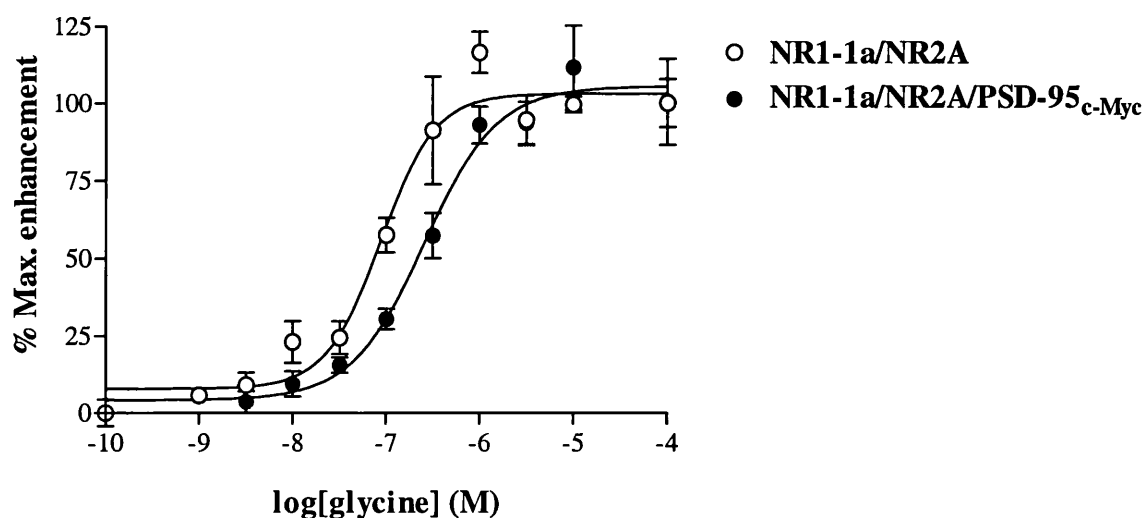


Figure 3.12 Enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} by glycine

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cells were harvested and washed extensively. The resulting cell homogenates (150 µg protein) were assayed for [³H]MK801 binding (2 nM) in the presence of 1 mM L-glutamate and a range of glycine concentrations (0 - 100 µM). The enhancement of [³H]MK801 specific binding by glycine at each point was calculated as the percentage of the control specific binding, *i.e.* [³H]MK801 binding measured in the absence of added glycine. It is expressed here as a percentage of the maximal enhancement induced by 100 µM glycine. The results shown are representative curves obtained from an experiment repeated at least three times. Data points are means ± SD (bars) of triplicate determinations from a single experiment. EC₅₀ values, percentage maximum enhancement determinations and Hill coefficients are summarised in Table 3.4. Typical increases in triplicate determinations for specific [³H]MK801 (2 nM) binding to NR1-1a/NR2A receptors induced by the addition of 100 µM glycine compared to control binding, *i.e.* in the absence of added glycine, were 756 ± 44 to 1960 ± 30 dpm in the presence of PSD-95_{c-Myc}, and 323 ± 4 to 842 ± 6 dpm in the absence of PSD-95_{c-Myc}.

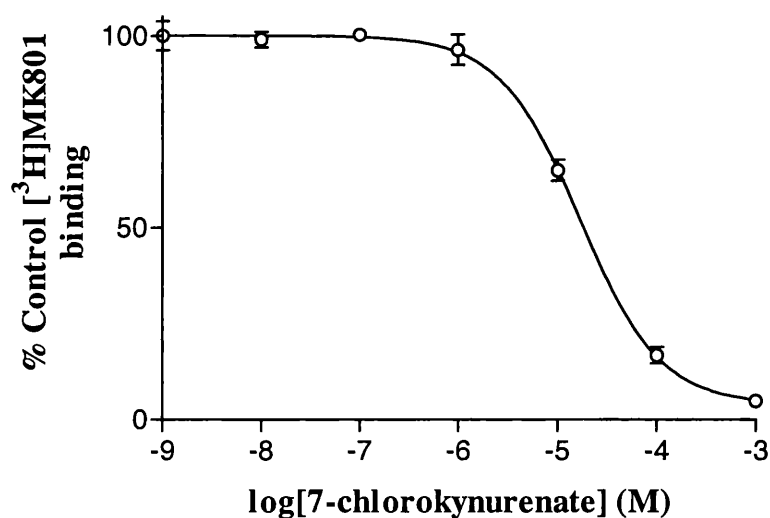


Figure 3.13 Inhibition of [³H]MK801 binding by 7-chlorokynurenate

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc}. Cells were harvested and washed extensively as described in 2.2.30.2 and cell homogenates (150 µg protein) assayed for [³H]MK801 binding (2 nM) in the presence of 1 mM L-glutamate, 10 µM glycine and a range of 7-chlorokynurenate concentrations (0 - 1 mM). Results are expressed as the percentage of control [³H]MK801 specific binding, *i.e.* [³H]MK801 binding measured in the absence of added 7-chlorokynurenate. Data points are means ± SD (bars) of triplicate determinations from a single transfection.

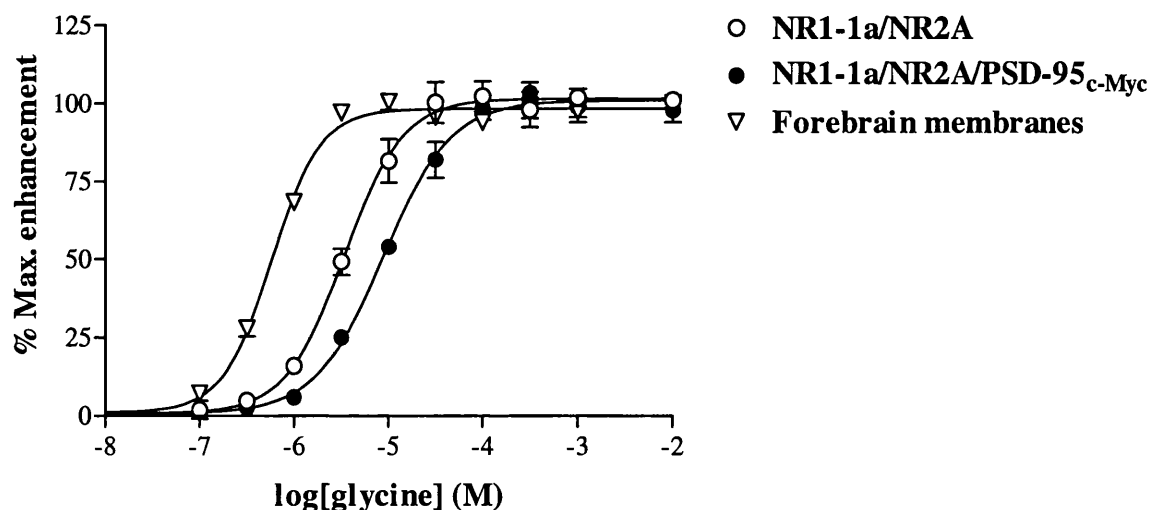


Figure 3.14 Enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} by glycine in the presence of 10 μM 7-chlorokynurenate

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cells were harvested and washed extensively. Cell homogenates (150 μg protein) and rat forebrain membranes (▽; 100 μg protein) were assayed for [³H]MK801 binding (2 nM) in the presence of 1 mM L-glutamate, 10 μM 7-chlorokynurenate and a range of glycine concentrations (0 - 10 mM). The enhancement of [³H]MK801 specific binding by glycine at each point was calculated as the percentage of the control specific binding, *i.e.* binding measured in the absence of added glycine. It is expressed here as a percentage of the maximum enhancement induced by 100 μM glycine. The results shown are representative curves from an experiment repeated at least three times. Data points are means ± SD (bars) of triplicate determinations from a single experiment. EC₅₀ values, percentage maximum enhancement determinations and Hill coefficients are summarised in Table 3.4. Typical increases in triplicate determinations for specific [³H]MK801 (2 nM) binding to NR1-1a/NR2A receptors induced by the addition of 100 μM glycine compared to control binding, *i.e.* in the absence of added glycine, were 269 ± 18 to 1870 ± 60 dpm in the presence of PSD-95_{c-Myc}, and 115 ± 21 to 1040 ± 20 dpm in the absence of PSD-95_{c-Myc}.

A. Modulation of [³H]MK801 binding by L-glutamate

Receptor preparation	EC ₅₀ (μM)		Maximum enhancement (%)		Hill coefficient	
NR1-1a/NR2A	1.8 ± 0.4	(5)	220 ± 60	(5)	1.45 ± 0.25	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	7.8 ± 1.7 ^a	(3)	240 ± 70	(3)	1.04 ± 0.10	(3)
Forebrain membranes	0.46 ± 0.04 ^{b/c}	(3)	130 ± 30	(3)	1.22 ± 0.06	(3)

^a*p*<0.001, ^b*p*<0.01, ^c*p*<0.01

B. Modulation of [³H]MK801 binding by glycine

Receptor preparation	7-CK	EC ₅₀ (nM)		Maximum enhancement (%)		Hill coefficient	
NR1-1a/NR2A	-	67 ± 30	(4)	68 ± 63	(5)	1.48 ± 0.17	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	-	270 ± 50 ^a	(5)	140 ± 80	(5)	1.33 ± 0.23	(5)
NR1-1a/NR2A	10 μM	3100 ± 700	(3)	720 ± 70	(3)	1.50 ± 0.17	(3)
NR1-1a/NR2A/PSD-95 _{c-Myc}	10 μM	7300 ± 1500 ^a	(3)	660 ± 210	(3)	1.21 ± 0.02	(3)
Forebrain membranes	10 μM	670 ± 60 ^{b/c}	(3)	510 ± 250	(3)	1.59 ± 0.08	(3)

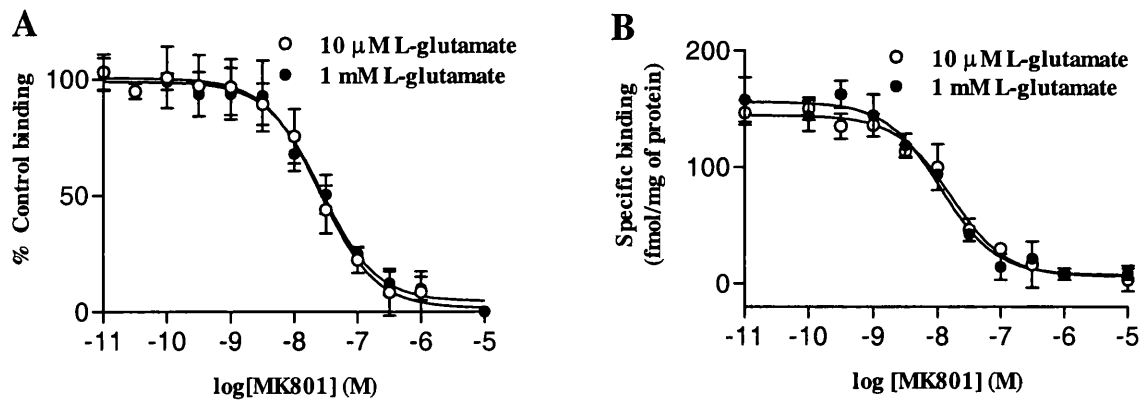
minus 7-CK: ^a*p*<0.001

plus 7-CK: ^a*p*<0.05, ^b*p*<0.01, ^c*p*<0.01

Table 3.4 Summary of the parameters for the enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} by L-glutamate and glycine

The dose-dependent enhancement curves for the modulation of [³H]MK801 binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} and rat forebrain membranes by L-glutamate (A) and glycine (B) were analysed by GraphPAD Prism software. EC₅₀ is the concentration of L-glutamate or glycine required to elicit 50% maximal enhancement of specific [³H]MK801 binding. The maximum level of enhancement elicited by L-glutamate or glycine is expressed as a percentage of control binding, *i.e.* Specific [³H]MK801 assayed in the absence of added L-glutamate or glycine. 7-CK is the concentration of 7-chlorokynurenate in the binding assay if appropriate. Values are the means ± SD of (n) determinations. Unpaired students *t* tests were used to compare the values obtained for NR1-1a/NR2A/PSD-95_{c-Myc} (^a) with those of NR1-1a/NR2A, and to compare the parameters obtained for rat forebrain membranes with those of NR1-1a/NR2A receptors in the presence (^b) and absence (^c) of PSD-95_{c-Myc}. Levels of significance for these comparisons are shown under each table.

MK801 binding to NR1-1a/NR2A receptors



MK801 binding to NR1-1a/NR2A receptors + PSD-95

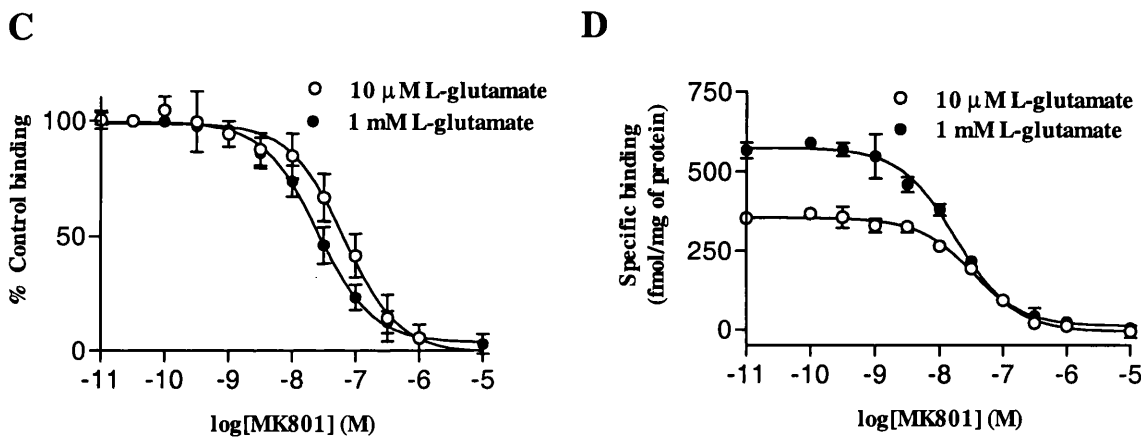
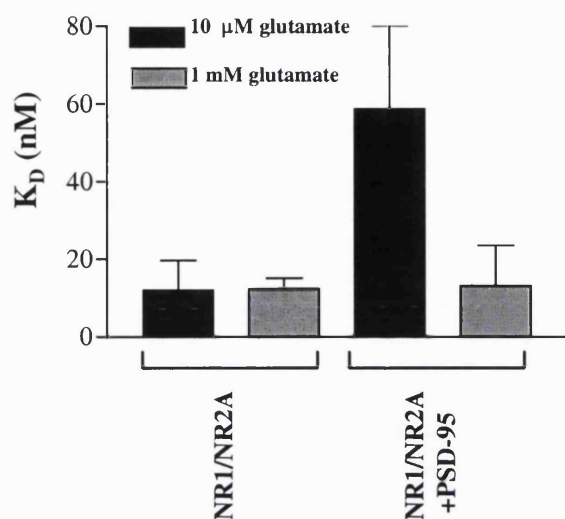


Figure 3.15 The effect L-glutamate on the affinity of NR1-1a/NR2A receptors for MK801 in the presence and absence of PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were assayed for [³H]MK801 binding (10 nM) in the presence of 30 μ M glycine and either 10 μ M L-glutamate or 1 mM L-glutamate. **A**, **B**, **C** and **D**. Displacement of [³H]MK801 binding by MK801 to NR1-1a/NR2A receptors in the presence (**C** and **D**) and absence (**A** and **B**) of PSD-95_{c-Myc} performed in the presence of either 10 μ M (○) or 1 mM (●) L-glutamate. Results are expressed as a percentage of control [³H]MK801 specific binding (**A** and **C**) and as specific [³H]MK801 binding (**B** and **D**). Data points in **A** and **C** are the combined means $\pm$ SD (bars) of triplicate determinations from at least $n = 2$ independent transfections. **B** and **D** are representative competition curves where data points are the means $\pm$ SD (bars) of triplicate determinations from a single transfection. Resulting K_i values are summarised in Figure 3.16. Typical triplicate determinations for control specific [³H]MK801 binding in the presence of 10 μ M L-glutamate and 1 mM L-glutamate were 1050 ± 20 and 1712 ± 60 dpm respectively for NR1-1a/NR2A receptors + PSD-95_{c-Myc} and 437 ± 26 and 442 ± 63 dpm for NR1-1a/NR2A receptors - PSD-95_{c-Myc}.

A**B**

Receptor Preparation	K _i (μM)		K _i (μM)	
	(+10 μM L-glutamate)		(+1 mM L-glutamate)	
NR1-1a/NR2A	12 ± 7	(5)	12 ± 2	(2)
NR1-1a/NR2A/PSD-95	59 ± 19	(5)	13 ± 9	(3)

Figure 3.16 The effect of L-glutamate on the parameters for MK801 binding to NR1-1A/NR2A receptors $\pm$ PSD-95_{c-Myc}

A and **B**. Histogram and table summarising the K_i values determined for the MK801 binding to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} in the presence of 30 μ M glycine and either 10 μ M or 1 mM L-glutamate. The results are means $\pm$ SD of (n) independent experiments or the means $\pm$ range of n = 2.

Receptor Preparation	Buffer	Specific [³ H]CGP39653 binding	Specific [³ H]CGP39653 binding (+ 7-CK)
NR1-1a/NR2A	1	77 ± 15	84 ± 16
NR1-1a/NR2A/PSD-95 _{c-Myc}	1	143 ± 3	191 ± 27
NR1-1a/NR2A	2	1710 ± 70	2810 ± 80
NR1-1a/NR2A/PSD-95 _{c-Myc}	2	4640 ± 330	8520 ± 190

Table 3.5 Optimization of [³H]CGP 39653 binding to NR1-1a/NR2A receptors

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Cells were harvested and processed by homogenisation in either Tris-citrate buffer ([Buffer 1], 50 mM Tris-citrate, pH 7.1 at 4°C, 5 mM EDTA, and 5 mM EGTA) using the method described in 2.2.30.2 or in Tris-HCl buffer ([Buffer 2], 5 mM Tris-HCl, pH 8.0 at 4°C) using the method described in 2.2.30.4. The resulting cell homogenates (100 µg protein) were assayed for [³H]CGP 39653 radioligand binding (6 nM) in the presence and absence of 10 µM 7-chlorokynurenate (7-CK), using 100 µM L-glutamate for the determination of non-specific binding. Results are expressed as specific [³H]CGP 39653 binding in dpm and are means ± SD of triplicate determinations from a single transfection experiment.

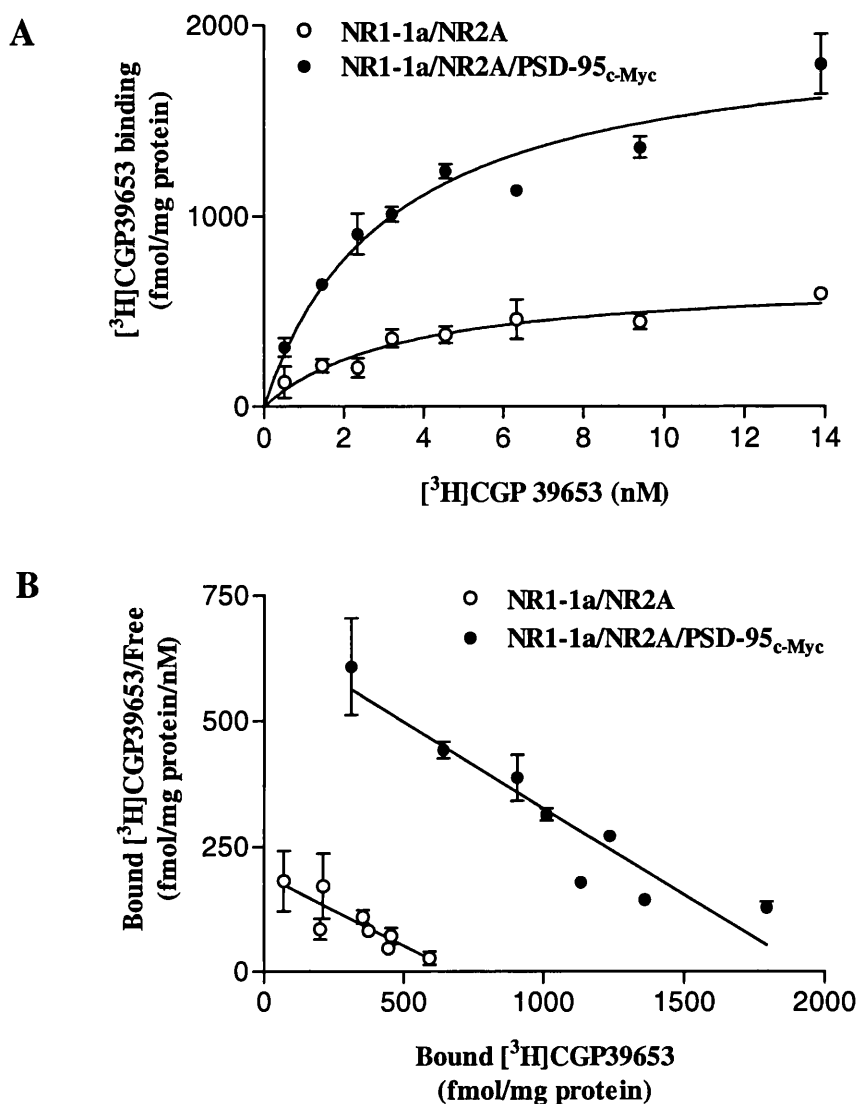


Figure 3.17 Saturation binding and Rosenthal transformation of $^{[3]H}$ CGP 39653 to NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cell homogenates were prepared as described in 2.2.30.4 and assayed for $^{[3]H}$ CGP 39653 radioligand binding in the presence of 10 μ M 7-chlorokynurenate with 100 μ M L-glutamate for the determination of non-specific binding. **A** and **B**. Representative saturation curves (**A**) and Rosenthal transformations (**B**) from an experiment repeated three times. Data points are means $\pm$ SD (bars) of triplicate determinations. Analysis of the curves by non-linear regression resolved a single binding site in both the presence and absence of PSD-95_{c-Myc}, with K_D and B_{max} values summarised in Table 3.6.

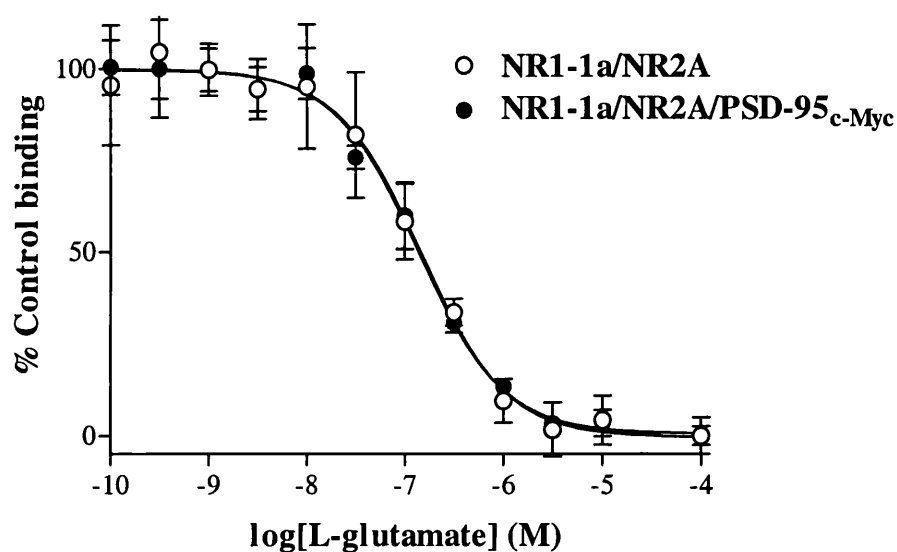


Figure 3.18 The effect of PSD-95_{c-Myc} on the affinity of NR1-1a/NR2A receptors for L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cell homogenates were prepared and assayed for [³H]CGP 39353 (6 nM) binding in the presence of 10 μM 7-chlorokynurenate and a 0 - 100 μM range of L-glutamate concentrations with 100 μM L-glutamate for the determination of non-specific binding. Results are expressed as a percentage of the control [³H]CGP 39653 specific binding, *i.e.* [³H]CGP 39653 binding assayed in the absence of added L-glutamate. The results presented are representative displacement curves from an experiment repeated three times. Data points are means ± SD (bars) of triplicate determinations from a single experiment. Analysis of the curves by non-linear regression (GraphPad Prism) resolved a single binding site both in the presence and absence of PSD-95_{c-Myc}, with K_i values and Hill coefficients summarised in Table 3.6. Typical total binding determinations in the absence of added L-glutamate were 7720 ± 340 and 3390 ± 280 dpm in the presence and absence of PSD-95_{c-Myc} respectively with corresponding non-specific binding determinations of 2070 ± 120 and 1870 ± 120.

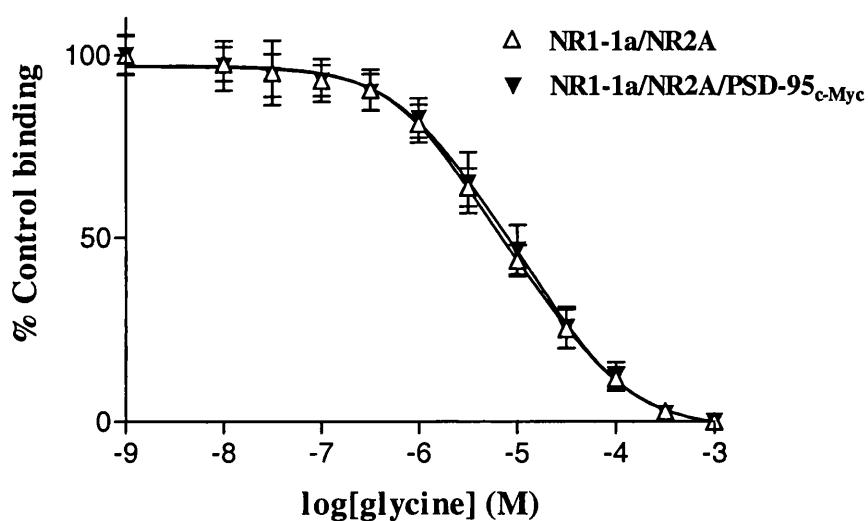


Figure 3.19 The effect of PSD-95_{c-Myc} on the affinity of NR1-1a/NR2A receptors for glycine

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (●) and absence (○) of PSD-95_{c-Myc}. Cell homogenates were prepared as described in 2.2.24 and assayed for [³H]MDL 105,519 binding in the presence of 1 mM L-glutamate and a 0 - 1 mM range of glycine with 1 mM glycine for the determination of non-specific binding. Results are expressed as a percentage of control [³H]MDL 105,519 specific binding. Data points are the combined means ± SD (bars) of triplicate determinations from n = 4 independent transfections. Analysis of the curves by non-linear regression resolved a two site fit for the displacement of [³H]MDL 105,519 binding by glycine. K_i values and Hill coefficients are summarised in Table 3.6.

A**[³H]CGP 39653 and L-glutamate binding parameters**

Receptor Preparation	K _D (nM)	B _{max} (fmol/mg protein)	K _I (nM)	Hill Coefficient
NR1-1a/NR2A	4.7 ± 0.6	760 ± 120	81 ± 13	1.03 ± 0.11
NR1-1a/NR2A/PSD-95 _{c-Myc}	6.6 ± 3.1	1800 ± 200	71 ± 18	0.90 ± 0.05

B**[³H]MDL 105,519 displacement by glycine**

Receptor Preparation	K _I site 1 (μM)	Fraction site 1 (%)	K _I site 2 (μM)	Hill Coefficient
NR1-1a/NR2A	1.9 ± 1.0	56 ± 16 %	34 ± 17	0.70 ± 0.07
NR1-1a/NR2A/PSD-95 _{c-Myc}	1.9 ± 1.3	49 ± 15 %	30 ± 19	0.71 ± 0.07

Table 3.6 Summary of the parameters for [³H]CGP 39653, L-glutamate and glycine binding to NR1-1A/NR2A receptors ± PSD-95_{c-Myc}

A. Table summarising the parameters for [³H]CGP 39653 and L-glutamate binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc}. K_D and B_{max} were determined by [³H]CGP39653 saturation binding studies. K_I values and the corresponding Hill coefficients were determined by L-glutamate displacement of [³H]CGP 39653 binding. Results are means from n = 3 independent experiments. **B.** Table summarising the parameters for the displacement of [³H]MDL 105,519 binding by glycine. Results are means from n = 4 independent experiments.

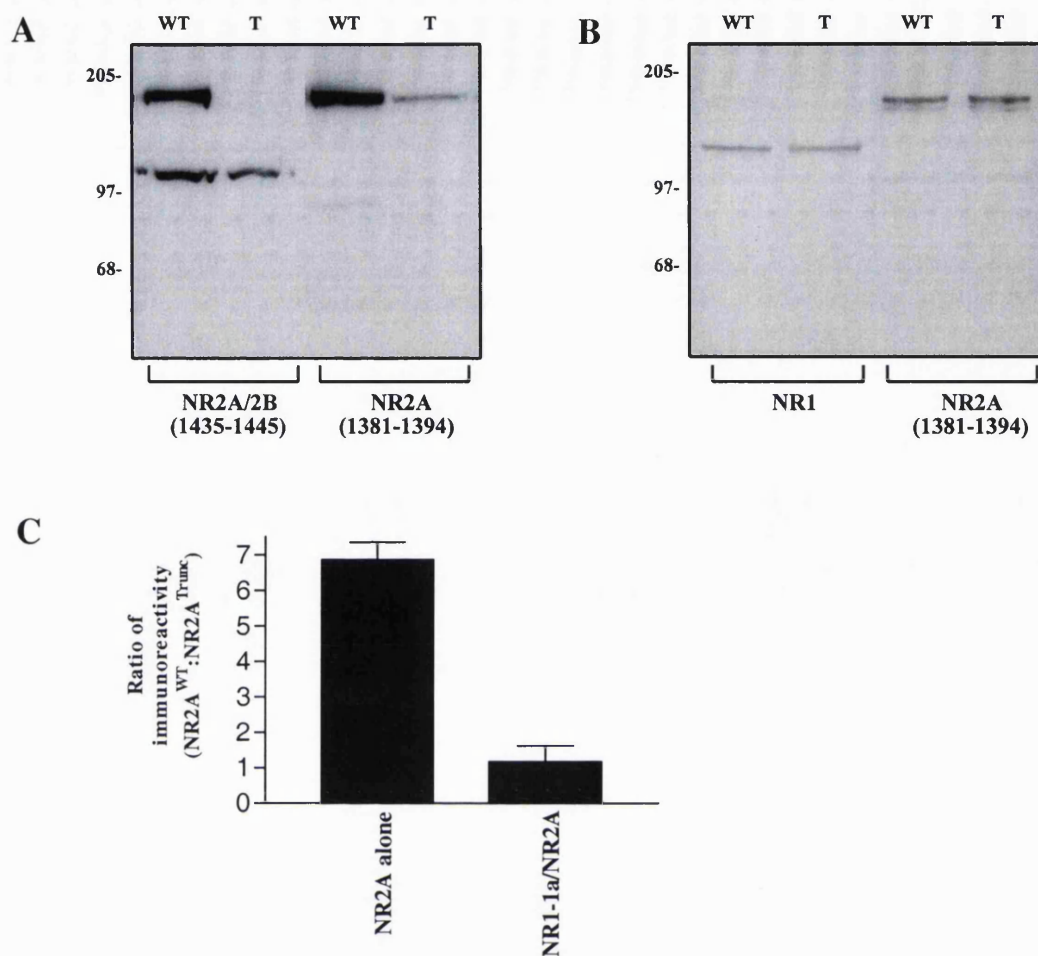


Figure 3.20 Immunoblots characterising the expression of NR2A^{Trunc} in HEK 293 cells

HEK 293 cells were transfected with either NR2A^{WT} and NR2A^{Trunc} alone or NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc}. Cell homogenates were analysed by quantitative immunoblotting using the following antibodies. **A.** HEK 293 cells transfected with NR2A^{WT} (WT) and NR2A^{Trunc} (T), immunoblotted with anti-NR2A/2B (1,435-1,445) and anti-NR2A (1,381-1,394) antibodies as indicated. **B.** HEK 293 cells transfected with NR1-1a/NR2A^{WT} (WT) or NR1-1a/NR2A^{Trunc} (T) immunoblotted with anti-NR1(911-920) and anti-NR2A (1,381-1,394) antibodies as indicated. In **A** and **B** the positions of the protein standards are shown on the left. **C.** Histogram summarising the results of the quantitative immunoblotting. The results are expressed as the ratio of the intensity of NR2A^{WT} immunoreactivities to the intensity of NR2A^{Trunc} immunoreactivities. Ratios are the mean $\pm$ SD of at least $n = 3$ separate transfections (for NR1/NR2 subunit combinations) or the mean $\pm$ range of $n = 2$ transfections (for NR2 alone) with $n = 3$ immunoblots per transfection.

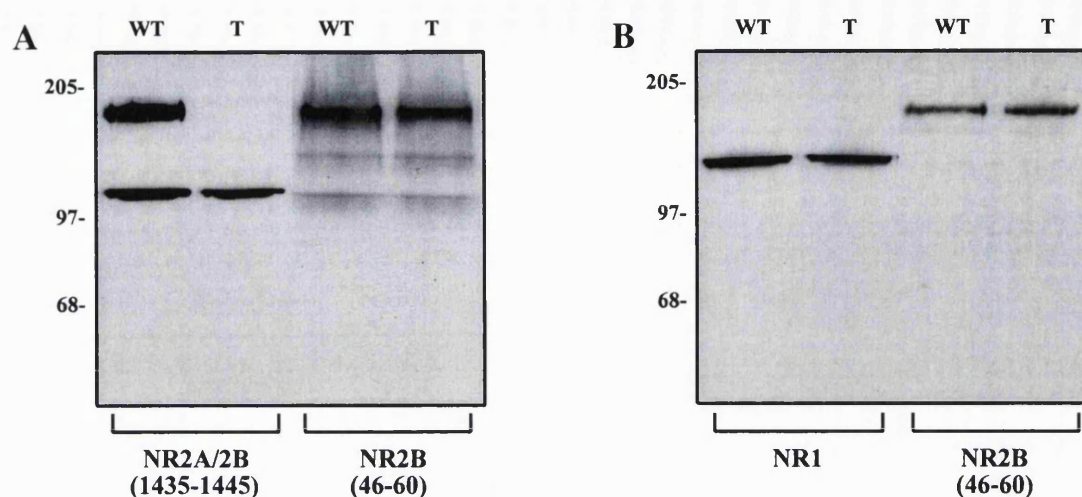


Figure 3.21 Immunoblots characterising the expression of NR2B^{Trunc} in HEK 293 cells

HEK 293 cells were transfected with either NR2B^{WT} and NR2B^{Trunc} alone or NR1-1a/NR2B^{WT} and NR1-1a/NR2B^{Trunc}. Cell homogenates were analysed by quantitative immunoblotting using the following antibodies. **A.** HEK 293 cells transfected NR2B^{WT} (WT) and NR2B^{Trunc} (T), immunoblotted with anti-NR2A/2B (1,435-1,445) and anti-NR2B (46-60) antibodies as indicated. **B.** HEK 293 cells transfected with NR1-1a/NR2B^{WT} (WT) or NR1-1a/NR2B^{Trunc} (T) immunoblotted with anti-NR1(911-920) and anti-NR2B (46-60) antibodies as indicated. In **A** and **B** the positions of the protein standards are shown on the left.

Receptor	K_D		B_{max}		EC_{50}	
Preparation	(nM)		(fmol/mg protein)		(μM)	
NR1-1a/NR2A ^{WT}	12 ± 7	(5)	680 ± 60	(5)	1.8 ± 0.4	(4)
NR1-1a/NR2A ^{Trunc}	13 ± 1	(3)	570 ± 70	(4)	1.5	(1)

Table 3.7 Summary of the parameters for [³H]MK801 radioligand binding to NR1-1a/NR2A^{Trunc} receptors

NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors were characterised by [³H]MK801 radioligand binding. K_D and B_{max} are the dissociation constant and number of [³H]MK801 binding sites determined by direct binding respectively. EC_{50} is the concentration of L-glutamate required to elicit 50% maximal enhancement of specific [³H]MK801 binding. Values are mean ± SD values determined from at (n) independent transfections

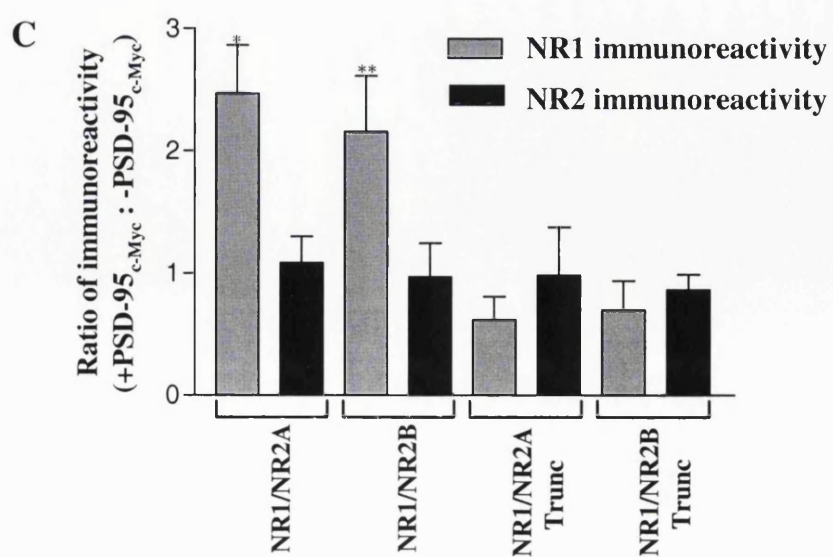
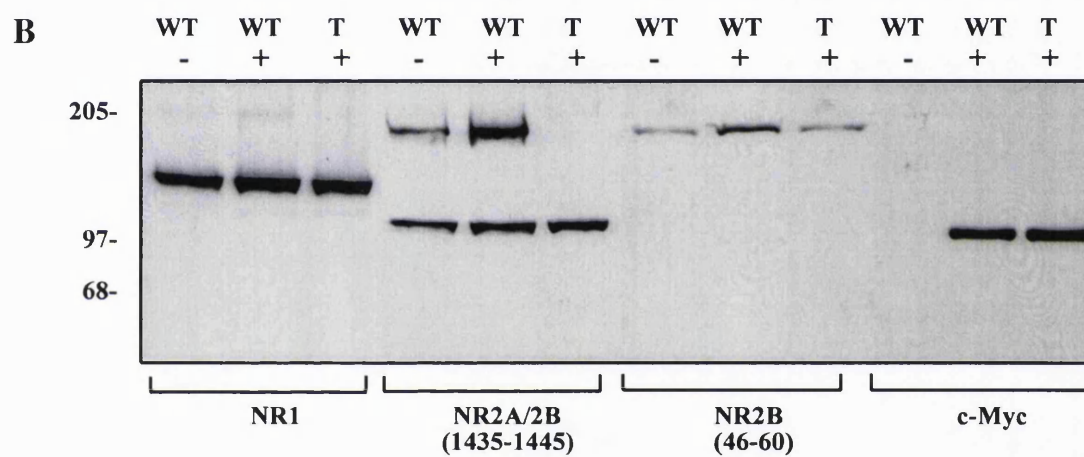
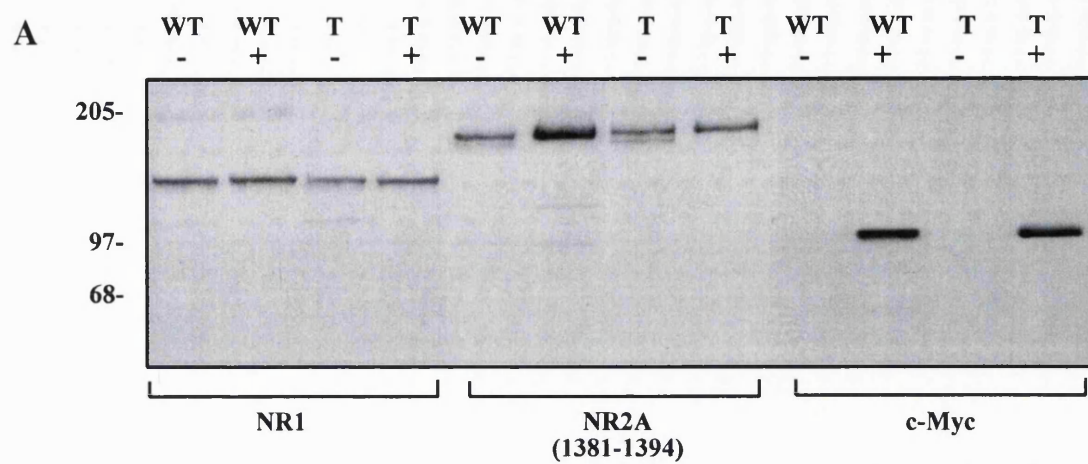
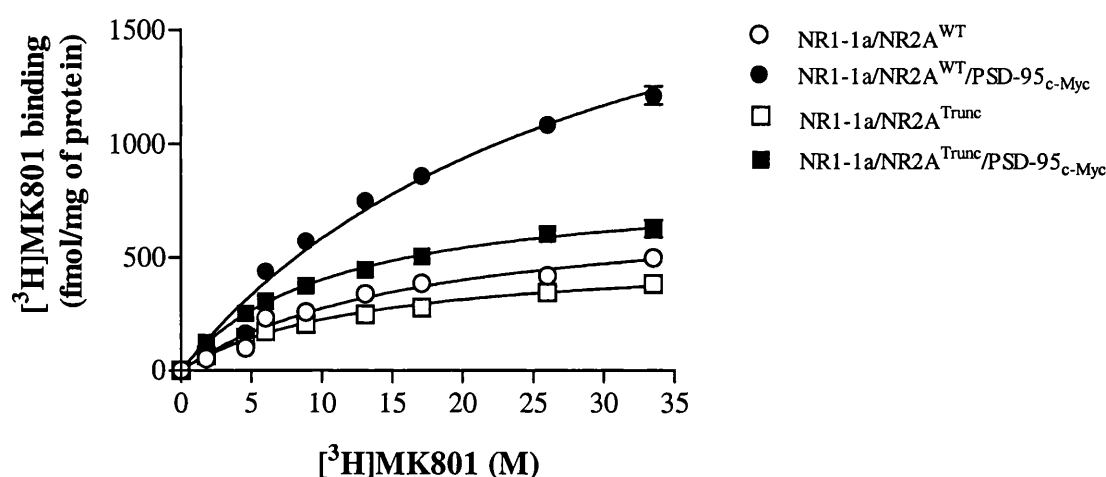


Figure 3.22 Immunoblots demonstrating the effect of PSD-95_{c-Myc} on the expression of C-terminally truncated NR2 subunits

HEK 293 cells were transfected with the following NMDA receptor subunit clones in the presence (+) and absence (-) of PSD-95_{c-Myc} and the resulting cell homogenates were analysed by quantitative immunoblotting with appropriate subunit specific antibodies. **A.** HEK 293 cells transfected with wild-type (W) NR1-1a/NR2A receptors or truncated (T) NR1-1a/NR2A^{Trunc} receptors in the presence (+) and absence (-) of PSD-95_{c-Myc} using anti-NR1 (911-920), anti NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. **B.** HEK 293 cells transfected with wild type (W) NR1-1a/NR2B receptors in the presence (+) and absence (-) of PSD-95_{c-Myc} or truncated (T) NR1-1a/NR2B^{Trunc} receptors in the presence (+) of PSD-95_{c-Myc} using anti-NR1 (911-920), anti-NR2A/B (1,435-1,445), anti-NR2B (46-60) and anti-c-Myc antibodies. In **A** and **B** the positions of the protein standards are shown on the left. **C.** Histogram summarising the results of the quantitative immunoblotting. Results are expressed as the ratio of immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to intensities measured in the absence of PSD-95_{c-Myc}. Ratios are means $\pm$ SD of at least $n = 3$ separate transfections with $n = 2$ immunoblots per transfection. The effect of PSD-95_{c-Myc} on the NMDA receptor subunit immunoreactive intensities was assessed using paired students t tests ($*p < 0.005$, $**p < 0.05$).

A



B

Receptor Preparation	K_D (nM)		B_{max} (fmol/mg of protein)	
NR1-1a/NR2A ^{WT}	17 ± 5	(5)	680 ± 60	(5)
NR1-1a/NR2A ^{WT} /PSD-95 _{c-Myc}	25 ± 4	(5)	2200 ± 400 ^a	(5)
NR1-1a/NR2A ^{Trunc}	13 ± 1	(3)	570 ± 70	(3)
NR1-1a/NR2A ^{Trunc} /PSD-95 _{c-Myc}	12 ± 1	(3)	880 ± 110 ^b	(3)

^a $p < 0.005$, ^b $p < 0.05$.

Figure 3.23 Saturation binding of [³H]MK801 to NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors ± PSD-95_{c-Myc}

HEK 293 cells were transfected with NR1-1a/NR2A^{WT} and NR1-1a/NR2A^{Trunc} receptors in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were assayed for [³H]MK801 radioligand binding in the presence of 30 μ M glycine and 1 mM L-glutamate. **A.** Representative saturation curves for specific [³H]MK801 binding to NR1-1a/NR2A^{WT} receptors in the presence (●) and absence (○) of PSD-95_{c-Myc} and to NR1-1a/NR2A^{Trunc} receptors in the presence (■) and absence (□) of PSD-95_{c-Myc}. Analysis of the curves by non-linear regression resolved single, high affinity binding sites in all cases with binding parameters summarised in **B.** Table summarising the B_{max} and K_D values from at least $n = 3$ independent transfections. The effect of PSD-95_{c-Myc} on the B_{max} values for binding to either NR1-1a/NR2A^{WT} (^a) or NR1-1a/NR2A^{Trunc} (^b) was assessed by paired students t tests. Levels of significance are shown below the table.

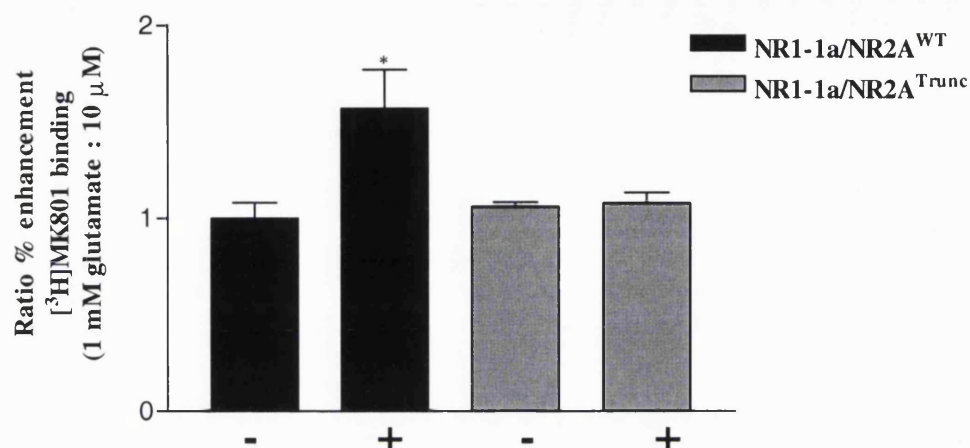


Figure 3.24 The effect of the NR2A C-terminal truncation on the PSD-95_{c-Myc}-induced decrease in the sensitivity of NR1-1a/NR2A receptors to L-glutamate

HEK 293 cells were transfected with either NR1-1a/NR2A^{WT} or NR1-1a/NR2A^{Trunc} receptors both in the presence (+) and absence (-) of PSD-95_{c-Myc}. Cells were harvested and washed extensively. The resulting cell homogenates were assayed for $[^3\text{H}]\text{MK801}$ binding (2 nM) in the presence of 30 μM glycine and either 0, 10 μM , or 1 mM L-glutamate. The enhancement of $[^3\text{H}]\text{MK801}$ binding at each point was calculated as the percentage of control specific binding, *i.e.* $[^3\text{H}]\text{MK801}$ binding assayed in the absence of added L-glutamate. Results are expressed as the ratios of the percentage enhancement at 1 mM L-glutamate to the percentage enhancement at 10 μM L-glutamate. Triplicate determinations were carried out for each concentration. The results are means $\pm$ SD (bars) of at least $n = 3$ independent experiments. Deviations of data set means from unity were analysed by unpaired students *t* tests (* $p < 0.0001$).

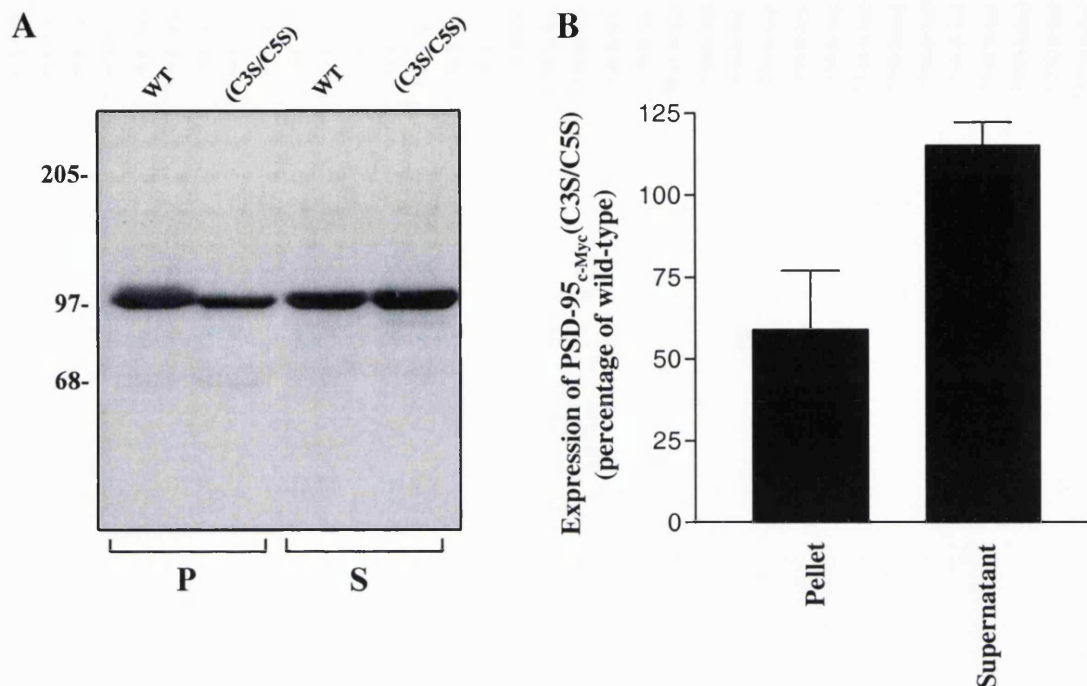


Figure 3.25 Characterisation of the expression of PSD-95_{c-Myc}(C3S/C5S) in HEK 293 cells

HEK 293 cells were transfected with either wild-type PSD-95_{c-Myc} (WT) or mutant PSD-95_{c-Myc}(C3S/C5S). Cells were harvested and processed by a single cycle of homogenisation and centrifugation. The pellets (P) and the soluble cytoplasmic fractions (S) were analysed by quantitative immunoblotting using the anti-c-Myc antibody. The immunoblot shown in **A** is representative of an experiment repeated at least twice. The positions of the molecular weight standards are shown on the left. **B**. Histogram summarising the results of the quantitative immunoblotting. The intensities of PSD-95_{c-Myc}(C3S/C5S) immunoreactive bands are expressed as percentages of the intensities of wild-type PSD-95_{c-Myc} immunoreactive bands. Results are the mean $\pm$ SD (bars) of at least $n = 3$ (pellet) independent transfections or the mean $\pm$ range (bars) of $n = 2$ (supernatant).

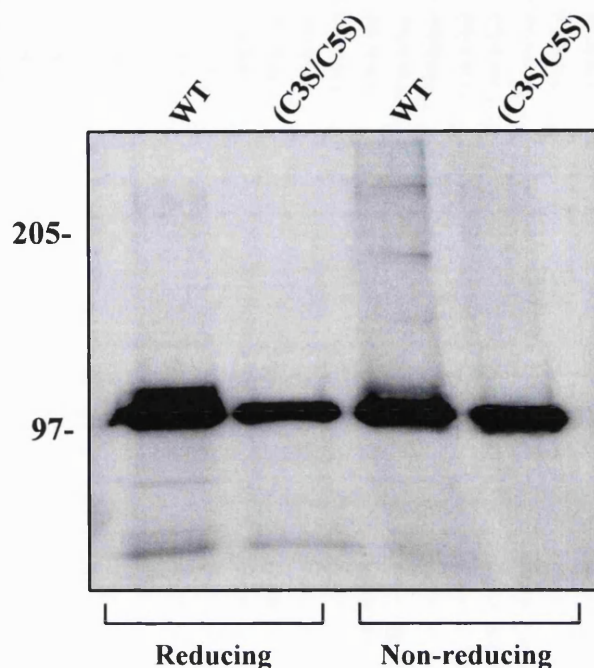


Figure 3.26 The analysis of transfected HEK 293 cell homogenates expressing PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) under reducing and non-reducing conditions

HEK 293 cells expressing either wild-type PSD-95_{c-Myc} (WT) or mutant PSD-95_{c-Myc}(C3S/C5S) were analysed by immunoblotting using anti-c-Myc antibodies under both reducing and non-reducing conditions as described in section 3.18. The positions of the molecular weight standard are shown on the left. The immunoblot is from a single experiment.

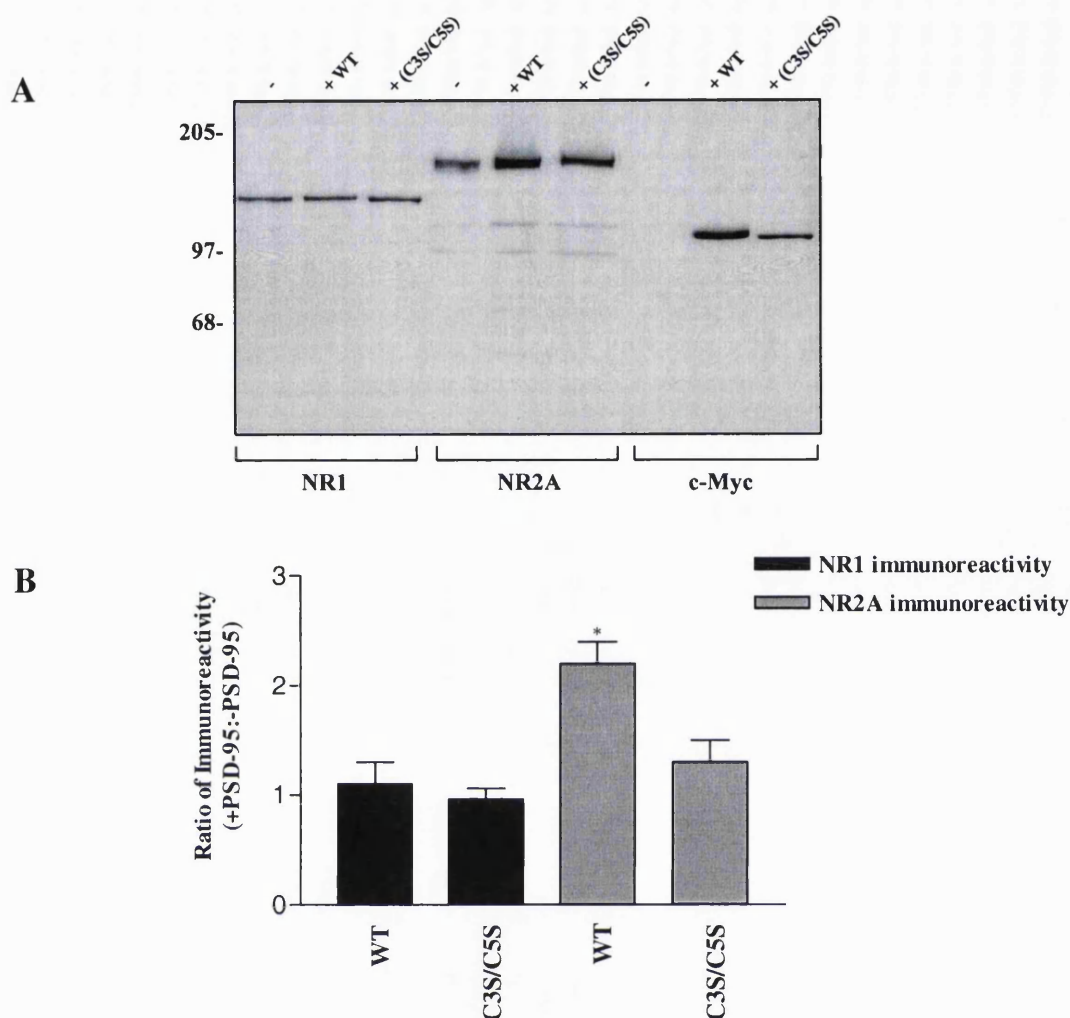


Figure 3.27 Immunoblots comparing the effects of PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) on the level of NR1-1a/NR2A receptor expression in HEK 293 cells

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (+) and absence (-) of either PSD-95_{c-Myc} (WT) or mutant PSD-95_{c-Myc}(C3S/C5S). The resulting cell homogenates were analysed by quantitative immunoblotting using anti-NR1 (911-920), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. Representative immunoblots are shown in **A**. The positions of the protein standards are shown on the left. **B**. Histogram summarising the results of the quantitative immunoblotting. Results are expressed as the ratio of the intensity of NR1 or NR2A immunoreactivities measured in the presence of either PSD-95_{c-Myc} (WT) or mutant PSD-95_{c-Myc}(C3S/C5S) to the intensities measured in their absence. Ratios are means $\pm$ SD (bars) of $n = 3$ independent transfections with $n = 3$ immunoblots per transfection. Fold changes in immunoreactive intensities were analysed by paired students t tests ($*p < 0.01$).

A

Receptor Preparation	Single point (fmol/mg of protein)		K _D (nM)	
NR1-1a/NR2A	420 ± 60	(3)	17 ± 5	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	1200 ± 80 ^a	(3)	25 ± 4	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc} (C3S/C5S)	790 ± 100 ^{b/c}	(3)	24	(1)

^a*p* < 0.005, ^b*p* < 0.05, ^c*p* < 0.005.

B

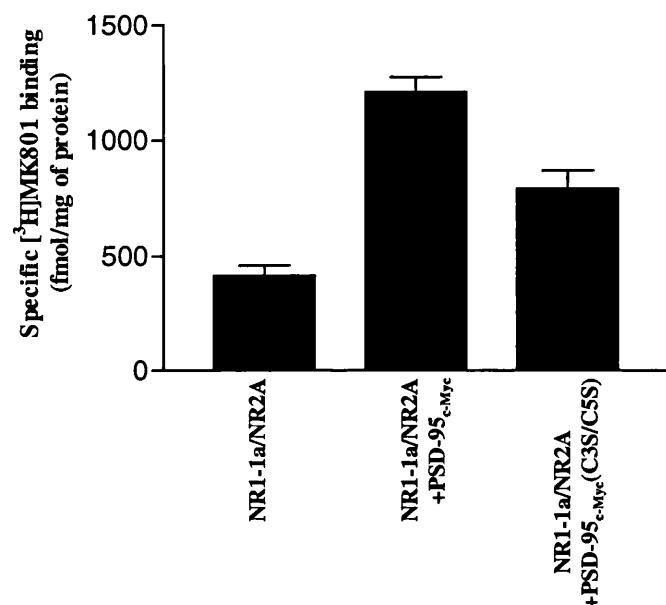


Figure 3.28 A comparison of the effects of PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) on single point [³H]MK801 binding to NR1-1a/NR2A^{WT} receptors

HEK 293 cells were transfected with NR1-1a/NR2A^{WT} in the presence and absence of PSD-95_{c-Myc} and in the presence of PSD-95_{c-Myc}(C3S/C5S). The resulting cell homogenates were assayed for [³H]MK801 binding (40 nM) in the presence of 30 μM glycine and 1 mM L-glutamate. **A.** Table summarising the specific [³H]MK801 binding to each receptor combination. Triplicate determinations were carried out per transfection. The results are the means ± SD from *n* = 3 independent transfections. The K_D values were determined by [³H]MK801 saturation binding. The results are means ± SD of (*n*) independent transfections. **B.** Histogram comparing the single point [³H]MK801 binding to each receptor combination. Paired students *t* tests were used to compare the single point value for both NR1-1a/NR2A/PSD-95_{c-Myc} (^a) with that of NR1-1a/NR2A, and NR1-1a/NR2A/PSD-95_{c-Myc}(C3S/C5S) with those of NR1-1a/NR2A receptors in the presence (^b) and absence (^c) of PSD-95_{c-Myc}. Levels of significance for these comparisons are shown under each table.

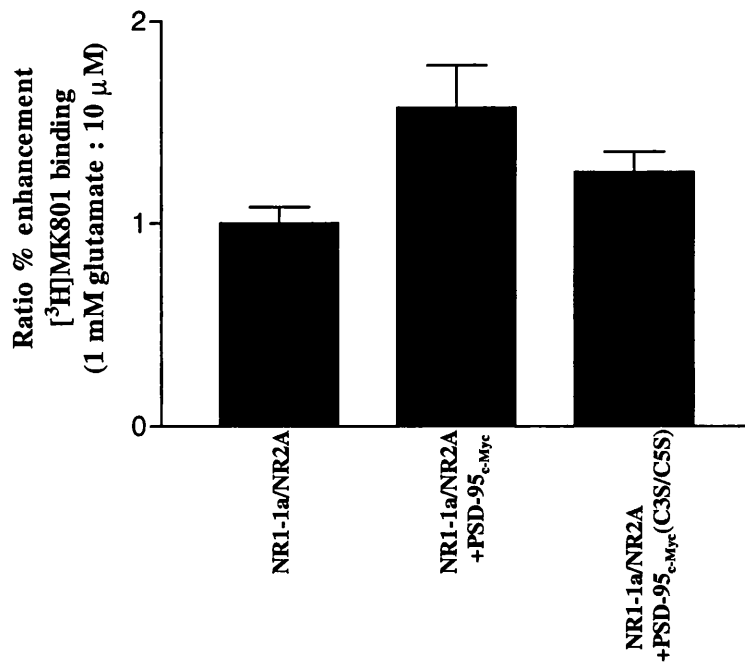


Figure 3.29 A comparison of the effects of PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) on the sensitivity of NR1-1a/NR2A receptors to L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A^{WT} receptors both in the presence and absence of PSD-95_{c-Myc} and in the presence of PSD-95_{c-Myc}(C3S/C5S). Cells were harvested and washed extensively. The resulting cell homogenates were assayed for [³H]MK801 binding (2 nM) in the presence of 30 μM glycine and either 0, 10 μM, or 1 mM L-glutamate. The enhancement of [³H]MK801 binding at each point was calculated as the percentage of the control specific binding, *i.e.* [³H]MK801 binding assayed in the absence of added L-glutamate. Results are expressed as the ratios of the percentage enhancement at 1 mM L-glutamate to the percentage enhancement at 10 μM. Triplicate determinations were carried out for each concentration. Ratios are means ± SD (bars) of at least n = 3 independent experiments. The statistical analysis of these data sets is referred to in the main text.

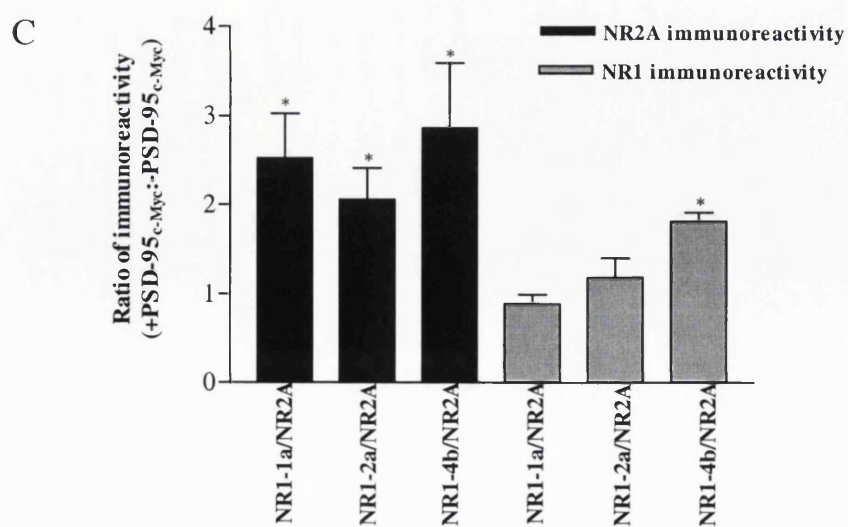
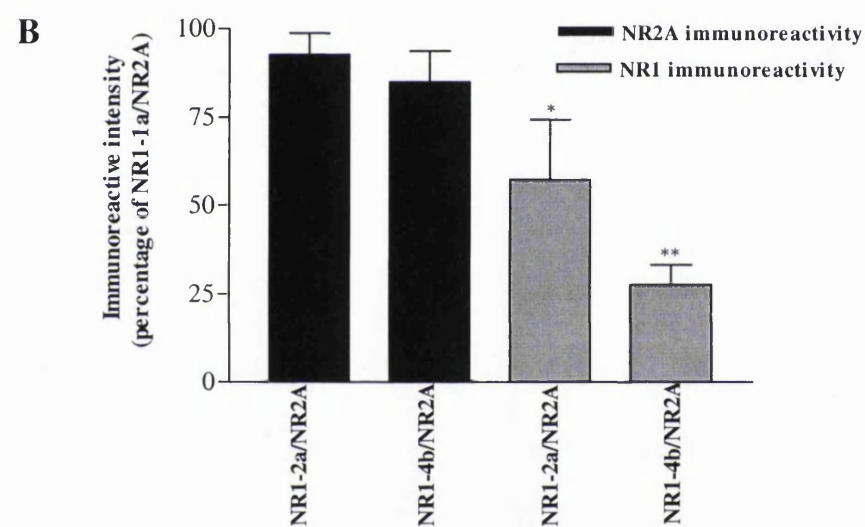
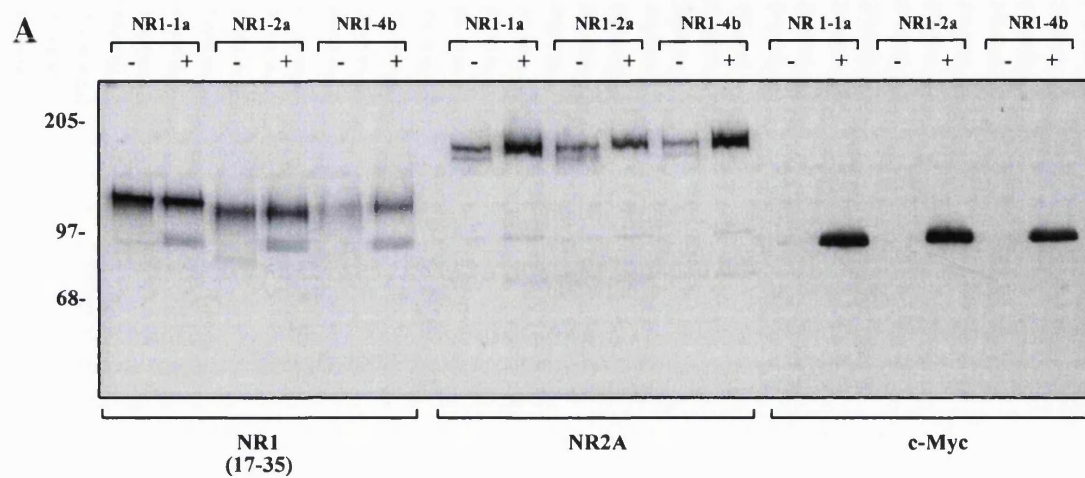


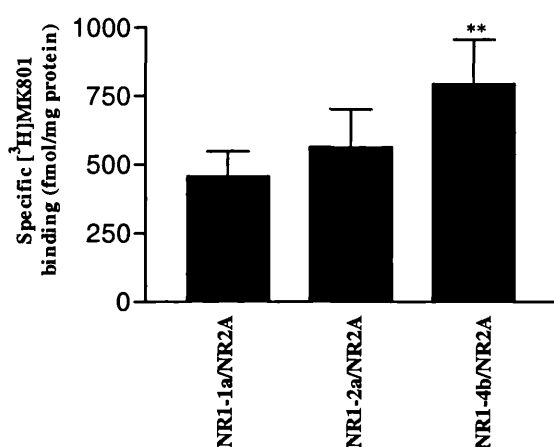
Figure 3.30 Quantitative analysis of the expression NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors $\pm$ PSD-95_{c-Myc} in HEK 293

HEK 293 cells were transfected with either NR1-1a/NR2A (labelled as NR1-1a in **A**), NR1-2a/NR2A (NR1-2a) or NR1-4b/NR2A (NR1-4b) receptor clones in the presence (+) and absence (-) of PSD-95_{c-Myc}. The resulting cell homogenates were analysed by quantitative immunoblotting. **A**. HEK 293 cell homogenates analysed by immunoblotting with anti-NR1 (46-60) and anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. The immunoblot is representative of an experiment repeated at least three times. The positions of the molecular weight markers are shown on the left. **B** and **C**. Histograms summarising the results of the quantitative immunoblotting. In **B**, the expression of NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors is compared. The intensities of both the NR1 and NR2A immunoreactive bands corresponding to NR1-2a/NR2A and NR1-4b/NR2A receptors, are expressed as a percentage of the immunoreactive intensities corresponding to NR1-1a and NR2A subunits respectively, expressed in an NR1-1a/NR2A subunit combination. In **C**, the effect of PSD-95_{c-Myc} on the level of NR1 and NR2A subunit expression is compared between the three splice variants. The results are expressed as the ratio of the immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to the intensities measured in the absence of PSD-95_{c-Myc}. Values in **B** and **C** are means $\pm$ SD (bars) of at least $n = 3$ independent transfections with $n = 2$ immunoblots per transfection. Fold changes in immunoreactive intensities were analysed by paired students t tests ($*p < 0.05$, $**p < 0.005$).

A

Receptor Preparation	Single point [^3H]MK801 binding (fmol/mg protein)
NR1-1a/NR2A	460 $\pm$ 90
NR1-1a/NR2A/PSD-95 _{c-Myc}	1400 $\pm$ 100
NR1-2a/NR2A	560 $\pm$ 140
NR1-2a/NR2A/PSD-95 _{c-Myc}	1100 $\pm$ 200
NR1-4b/NR2A	790 $\pm$ 160
NR1-4b/NR2A/PSD-95 _{c-Myc}	1100 $\pm$ 120

B



C

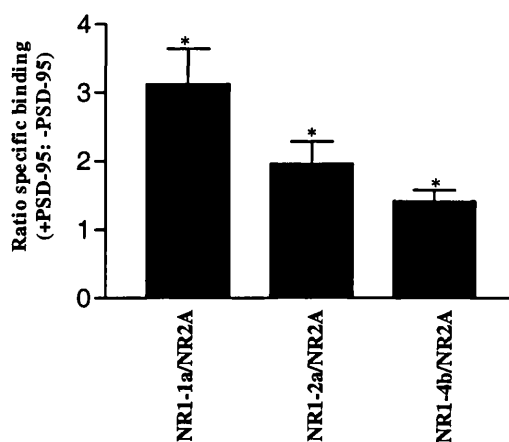


Figure 3.31 Single point [^3H]MK801 binding to NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors $\pm$ PSD-95_{c-Myc}

HEK 293 cells were transfected with either NR1-1a/NR2A, NR1-2a/NR2A or NR1-4b/NR2A receptor clones in the presence (+) and absence (-) of PSD-95_{c-Myc}. Cell homogenates were prepared and assayed for [^3H]MK801 binding (40 nM) in the presence of 1 mM L-glutamate and 30 μM glycine using 100 μM TCP for the determination of non-specific binding. **A.** Table summarising the specific [^3H]MK801 binding to each receptor subtype. Triplicate determinations were carried out per transfection. Results are means $\pm$ SD (bars) of $n = 3$ independent transfections. **B.** Histogram comparing specific [^3H]MK801 binding between NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors. Single point values for NR1-2a/NR2A and NR1-4b/NR2A were assessed for significant variations from NR1-1a/NR2A (** $p < 0.05$, paired t test). **C.** Histogram showing the effect of PSD-95_{c-Myc} on the specific [^3H]MK801 binding to the three receptor subtypes. Results are expressed as the ratio of the specific [^3H]MK801 binding to receptors in the presence of PSD-95_{c-Myc} to the binding measured in the absence of PSD-95_{c-Myc}. The effect of PSD-95_{c-Myc} on each receptor subtype was analysed by paired students t tests (* $p < 0.05$).

Receptor preparation	K _D (nM)	
NR1-1a/NR2A	16 ± 5	(5)
NR1-1a/NR2A/PSD-95 _{c-Myc}	25 ± 4	(5)
NR1-2a/NR2A	17	(1)
NR1-2a/NR2A/PSD-95 _{c-Myc}	23	(1)
NR1-4a/NR2A	14	(1)
NR1-4a/NR2A/PSD-95 _{c-Myc}	17	(1)

Table 3.8 Summary of the dissociation constants for [³H]MK801 binding to NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptor ± PSD-95_{c-Myc}

The K_D values were determined by [³H]MK801 saturation binding to HEK 293 cells transfected with NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors ± PSD-95_{c-Myc} in the presence of 1 mM L-glutamate and 30 μM glycine, with 1 mM ketamine for the determination of non-specific binding. Analysis of the curves by non-linear regression resolved single binding sites in all cases. The results are means from (n) independent transfection experiments.

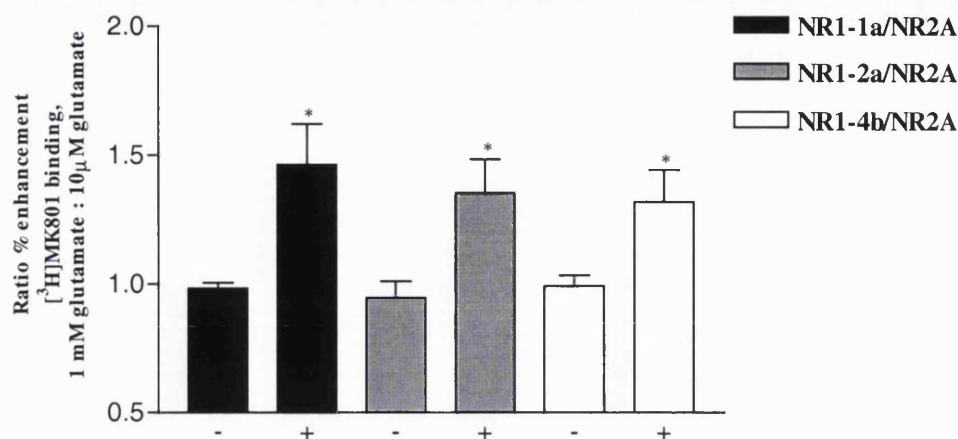


Figure 3.32 The effect of the NR1 splice form on the allosteric modulation of $[^3\text{H}]\text{MK801}$ binding to NR1/NR2A $\pm$ PSD-95_{c-Myc} by L-glutamate

HEK 293 cells were transfected with NR1-1a/NR2A, NR1-2a/NR2A and NR1-4b/NR2A receptors in the presence (+) and absence (-) of PSD-95_{c-Myc}. Cells were harvested and washed extensively. The resulting cell homogenates were assayed for $[^3\text{H}]\text{MK801}$ binding (2 nM) in the presence of 30 μM glycine and either 0, 10 μM , or 1 mM L-glutamate. The enhancement of $[^3\text{H}]\text{MK801}$ binding at each point was calculated as the percentage of the control specific binding, *i.e.* $[^3\text{H}]\text{MK801}$ binding measured in the absence of added L-glutamate. Results are expressed as the ratios of the percentage enhancement at 1 mM L-glutamate to the percentage enhancement at 10 μM . Triplicate determinations were carried out for each concentration and the ratios are means $\pm$ SD (bars) from $n = 3$ independent transfections. Significant deviations of data set means from unity were analysed by unpaired students t tests (* $p < 0.05$).

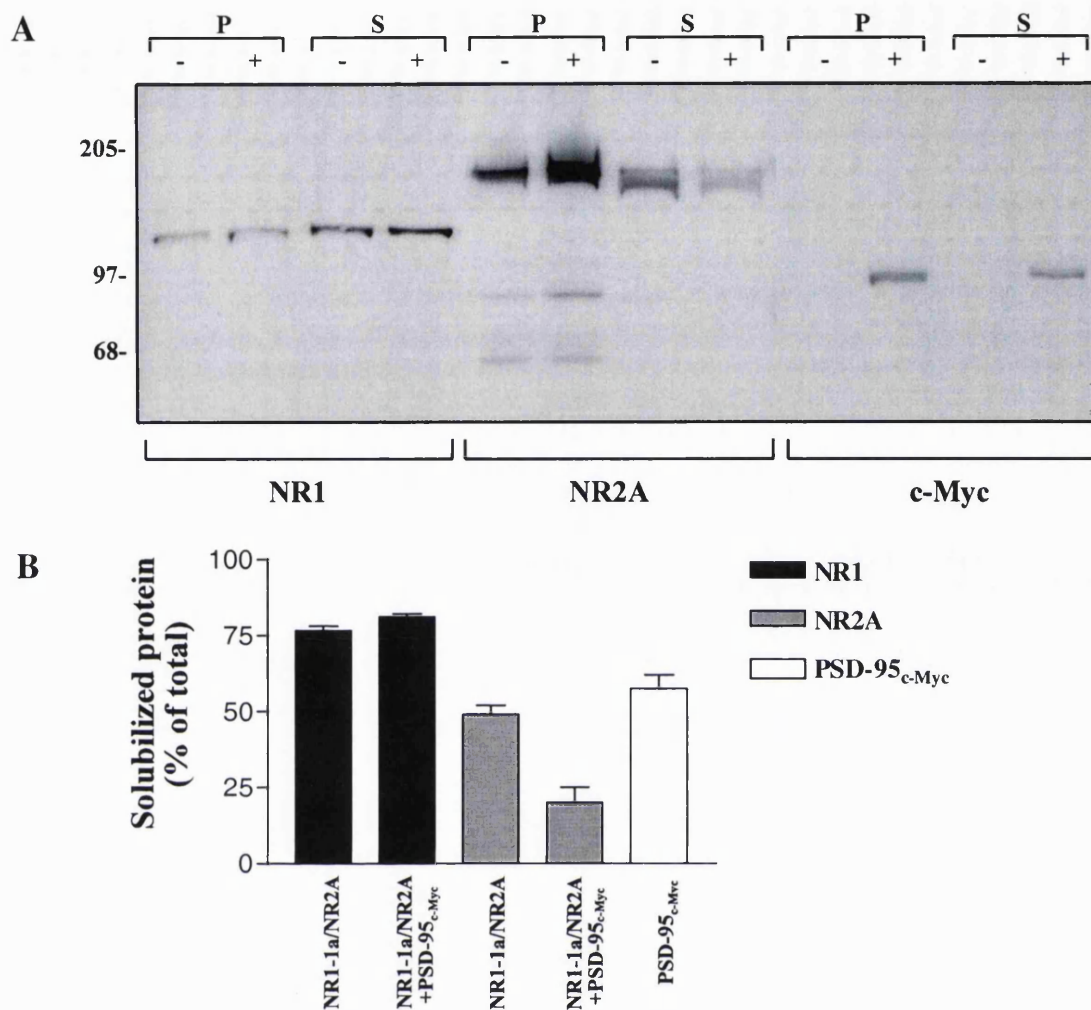


Figure 3.33 Immunoblots demonstrating the effect of the co-expression of PSD-95_{c-Myc} in HEK 293 cells on the detergent solubility of NR1-1a/NR2A receptors

HEK 293 cells transfected with NR1-1a/NR2A receptors in the presence (+) and absence (-) of PSD-95_{c-Myc} were detergent solubilized as described in 2.2.29. Samples of both the soluble fraction (S) and the insoluble pellet (P) were analysed by quantitative immunoblotting. **A.** Immunoblot analysis using anti-NR1 (911-920), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. Results are representative immunoblots from an experiment repeated twice. **B.** Histogram comparing the detergent solubility of NR1-1a and NR2A $\pm$ PSD-95_{c-Myc}. The intensities of NR2A and NR1-1a immunoreactivities present in the soluble fraction are expressed as a percentage of the total NR2A or NR1-1a immunoreactive intensities, *i.e.* soluble plus insoluble. The percentage solubility of PSD-95_{c-Myc} is also shown. Values are means $\pm$ range (bars) of $n = 2$ independent transfections.

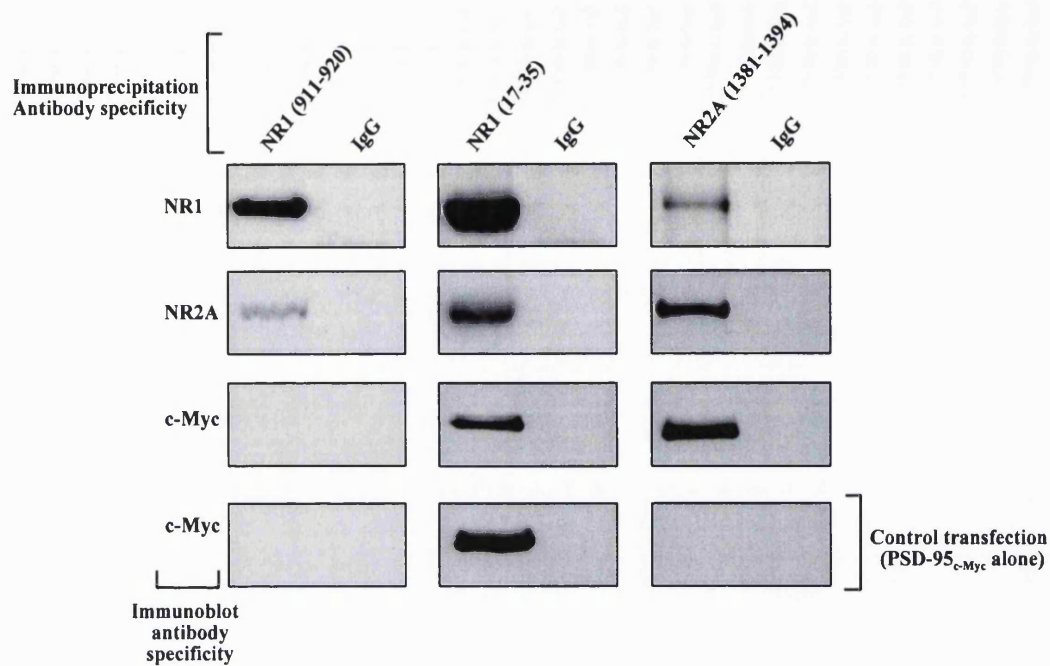


Figure 3.34 Characterisation of NMDA receptor subunit specific antibodies for the co-immunoprecipitation of NR1-1a/NR2A/PSD-95_{c-Myc} complexes

HEK 293 cells were transfected with either NR1-1a/NR2A/PSD-95_{c-Myc} or for control experiments, with PSD-95_{c-Myc} alone. The resulting cell homogenates were detergent solubilized and immunoprecipitation reactions were carried out using anti-NR1 (911-920), anti-NR1 (17-35) and anti-NR2A (1,381-1,394) antibodies as described in 2.2.29. Control immunoprecipitation reactions were performed using non-immune IgG. The resulting immune pellets were analysed by immunoblotting using anti-NR1 (911-920), anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated on the left. The immunoblots shown are representative of an experiment repeated at least twice.

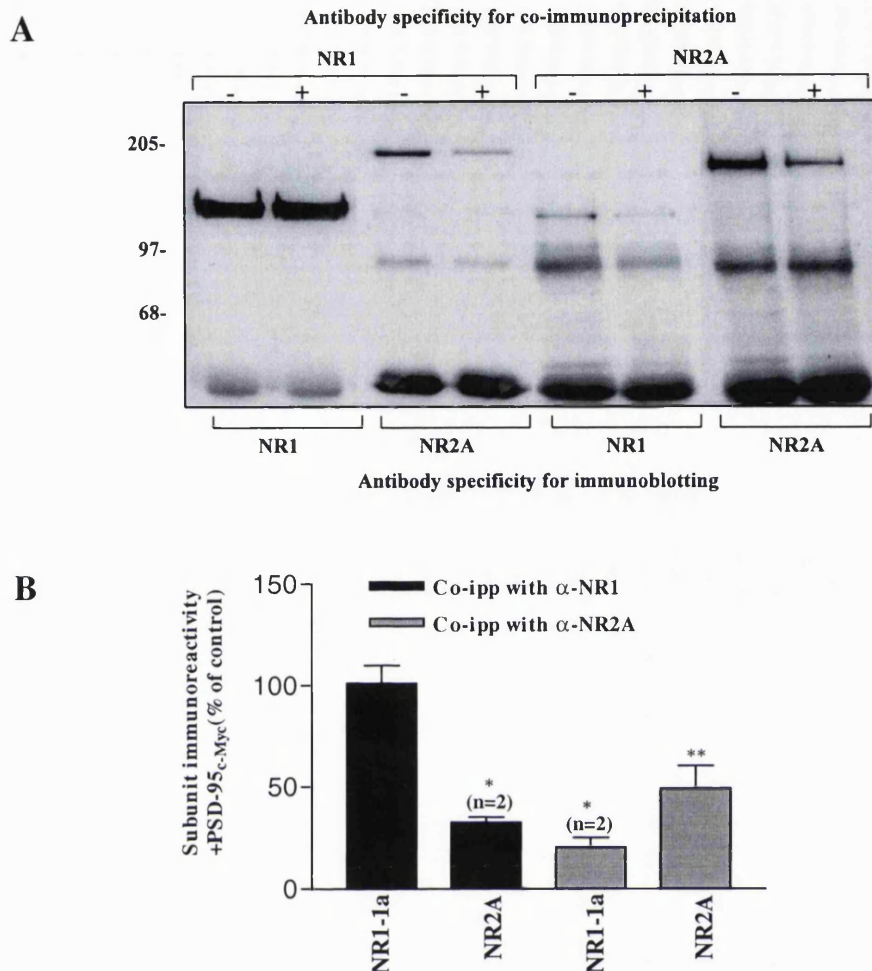


Figure 3.35 Immunoblots demonstrating the effect of PSD-95_{c-Myc} on the amount of NR1-1a and NR2A subunits immunoprecipitated from transfected HEK 293 cells

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (+) and absence (-) of PSD-95_{c-Myc}. Cell homogenates were detergent solubilized and immunoprecipitation reactions were performed using anti-NR2A (1,381-1,394) and anti-NR1 (911-920) antibodies. The resulting immune pellets were analysed by quantitative immunoblotting using anti-NR2A (1,381-1,394) and anti-NR1 (911-920) antibodies as indicated in **A**. The immunoblot shown is representative of an experiment repeated at least three times. **B**. Histogram summarising the results of the quantitative immunoblotting. The intensities of immunoreactive bands measured in the presence of PSD-95_{c-Myc} are expressed as a percentage of the intensities measured in control transfections, *i.e.* in the absence of PSD-95_{c-Myc}. The results are means $\pm$ SD (bars) of at least $n = 3$ independent transfections or the means $\pm$ range (bars) of $n = 2$. The effect of PSD-95_{c-Myc} on intensity of NR1 and NR2A subunit immunoreactivities was assessed using paired students *t* tests (* $p < 0.05$, ** $p < 0.0001$).

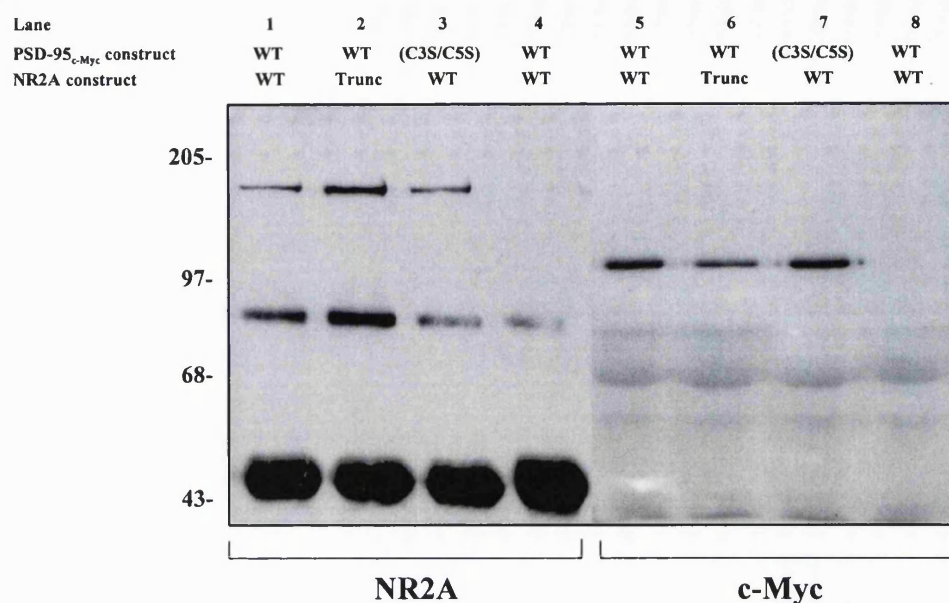


Figure 3.36 Immunoblots comparing the association of NR2A^{WT} and NR2A^{Trunc} with PSD-95_{c-Myc} and PSD-95_{c-Myc}(C3S/C5S) by co-immunoprecipitation studies

HEK 293 cells were transfected with either NR1-1a/NR2A^{WT}/PSD-95_{c-Myc}, NR1-1a/NR2A^{Trunc}/PSD-95_{c-Myc} or NR1-1a/NR2A^{WT}/PSD-95_{c-Myc}(C3S/C5S). Cell homogenates were detergent solubilized and immunoprecipitation reactions were performed using anti-NR2A (1,381-1,394) antibodies and for control experiments, non-immune IgG. Immune pellets were analysed by immunoblotting using anti-NR2A (1,381-1,394) and anti-c-Myc antibodies as indicated. Lanes 1 - 8 are labelled with respect to the combination of receptor/PSD-95 constructs present in the cell lysate. Lanes 4 and 8 are control co-immunoprecipitation reactions using non-immune IgG. The positions of the molecular weight standards are shown on the left. The results presented are representative immunoblots from an experiment repeated three times.

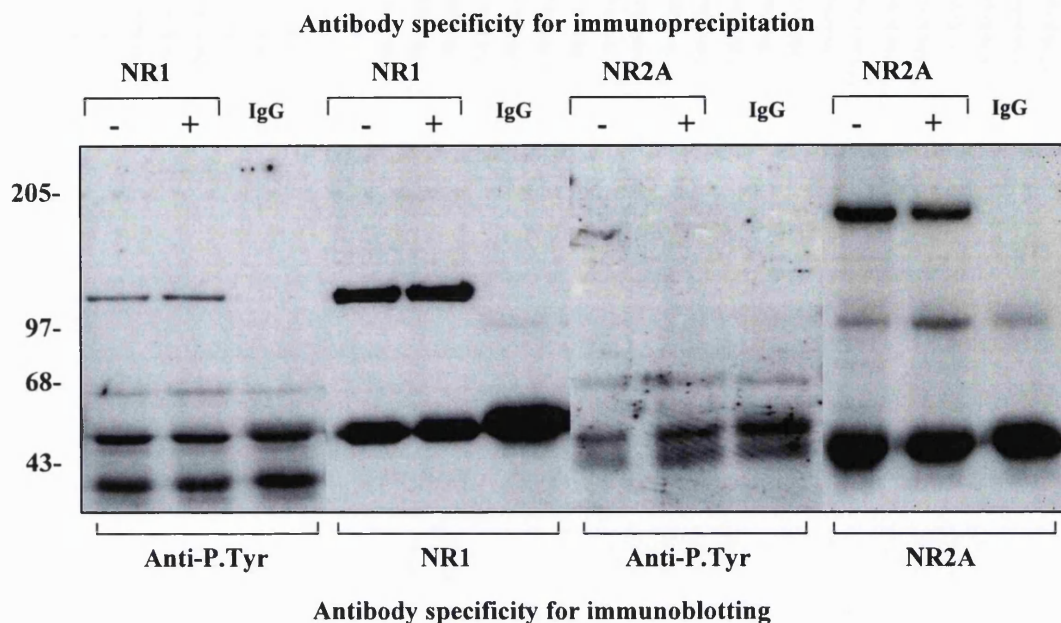


Figure 3.37 Analysis of NR1-1a and NR2A subunits by immunoblotting using anti-phosphotyrosine antibodies

HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence (+) and absence (-) of PSD-95_{c-Myc}. Cell homogenates were solubilized as described in 2.2.29. Immunoprecipitation reactions were then carried out using anti-NR1 (911-920), anti-NR2A (1,381-1,394) antibodies and for control reactions, non-immune IgG as indicated in the figure. Immune pellets were analysed by immunoblotting using anti-phosphotyrosine antibodies as described in 2.2.29 and then the nitrocellulose membranes were reprobed with anti-NR1 (911-920) and anti-NR2A (1,381-1,394) antibodies as indicated. The immunoblots shown are representative of an experiment repeated at least three times.

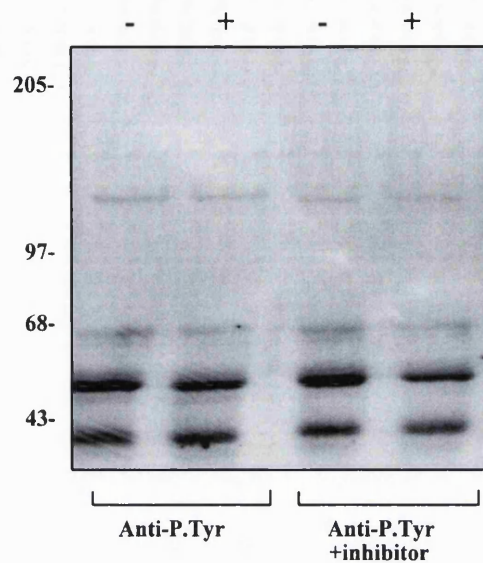


Figure 3.38 Investigation into the specificity of anti-phosphotyrosine antibodies

HEK 293 cells transfected with NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} were solubilized and immunoprecipitation reactions were carried out using anti-NR1 (911-920) antibodies. The resulting immune pellets were analysed by immunoblotting using anti-phosphotyrosine antibodies (anti-P.Tyr) as described in 2.2.29. The anti-phosphotyrosine antibodies were pre-incubated for 10 min with either PBS or phosphotyrosine inhibitor to test for antibody specificity against phosphorylated tyrosine residues. The immunoblot shown is from a single experiment.

CHAPTER 4

DISCUSSION

4.1 THE CO-EXPRESSION OF PSD-95 AND NMDA RECEPTORS IN HEK 293 CELLS

The results of this thesis comprise a detailed characterisation of the effects of PSD-95 on the biochemical and pharmacological properties of NMDA receptors expressed in HEK 293 cells. It was found that PSD-95 enhances the expression of NMDA receptors in HEK 293 cells as defined by quantitative immunoblotting and [³H]MK801 radioligand binding assays. Secondly, PSD-95 reduces the L-glutamate and glycine sensitivity of [³H]MK801 binding to NR1-1a/NR2A receptors. Thirdly, PSD-95 alters the biochemical properties of NR1-1a/NR2A receptors in transfected HEK 293 cells with respect to their detergent solubility. These results therefore, provide insights into the possible functional significance of the association of PSD-95 with NMDA receptors *in vivo*. Whereas most studies into the function of PSD-95 to date have focussed on the recruitment of additional regulatory and structural molecules to the post-synaptic membrane, these results implicate the direct association of PSD-95 with NMDA receptors in the regulation of channel function and expression in glutamatergic neurons. In addition, these results highlight the fact that cloned NMDA receptors expressed in non-neuronal cell lines are not necessarily representative of their native counterparts which may be highly modulated by the environment of the post-synaptic density.

4.2 THE EFFECT OF PSD-95 ON NMDA NR1 AND NR2 SUBUNIT EXPRESSION

Initial experiments investigated the effect of PSD-95 on the expression of the NR1-1a splice variant and NR2 subunits transfected alone or in heteromeric NR1-1a/NR2 subunit combinations. The results of these investigations are discussed in section 4.2.1. The effect of alternative NR1 subunit splice variants on the expression of NR1/NR2A receptors $\pm$ PSD-95_{c-Myc} was also characterised and is discussed in detail in section 4.2.2.

4.2.1 Enhanced expression of NR1-1a/NR2 receptors in the presence of PSD-95

Receptor Preparation	Total NR1	Total NR2	[³ H]MK801 B _{max}	[³ H]MDL105,519 B _{max}	[³ H]CGP39653 B _{max}
NR1-1a	↔	ND	ND	ND	ND
NR2A	ND	↑ ↑ ↑	ND	ND	ND
NR1-1a/NR2A	↔	↑ ↑ ↑	↑ ↑ ↑	↔	↑ ↑ ↑
NR1-1a/NR2B	↔	↑ ↑ ↑	ND	↔	ND
NR1-1a/NR2A ^{Trunc}	↔	↔	↑	ND	ND
NR1-1a/NR2B ^{Trunc}	↔	↔	ND	ND	ND

Table 4.1 A summary of the effects of PSD-95 on the expression of NR1-1a/NR2 receptors in HEK 293 cells

Total NMDA receptor subunit expression was determined by quantitative immunoblotting. ND is not performed. ↑, a PSD-95-induced increase in either immunoreactive intensities or B_{max} values. ↔, no effect.

Analysis of NMDA receptor expression by immunoblotting

The co-expression of PSD-95_{c-Myc} with NR1-1a or NR2A subunits alone resulted in an approximate three-fold increase in NR2A subunit immunoreactive intensities whereas no change in NR1-1a immunoreactive intensities was observed. When either NR2A or NR2B subunits were expressed in NR1-1a/NR2 subunit combinations, both wild-type PSD-95 and PSD-95_{c-Myc} again induced an approximate three-fold increase in NR2 subunit immunoreactivities, with no concomitant change in NR1-1a.

In order to determine whether this effect of PSD-95_{c-Myc} was dependent upon an interaction between PSD-95_{c-Myc} and the NR2 subunit, C-terminally truncated NR2A and NR2B subunits were generated that would not interact with PSD-95_{c-Myc}. A number of groups have reported that the NR2 subunit C-terminal ESDV amino acids are necessary for the association with PSD-95_{c-Myc} (Kornau *et al.*, 1995; Kim *et al.*, 1995; Bassand *et al.*, 1999). Therefore, it was expected that these truncated NR2 subunits would not interact with PSD-95_{c-Myc}. The initial characterisation of the expression of the NR2 mutants by quantitative immunoblotting revealed that the NR2B^{Trunc} subunit was expressed to the same level as NR2B^{WT}. The NR2A^{Trunc} subunit was also expressed to the same level as NR2A^{WT}.

when expressed in NR1-1a/NR2A subunit combinations but when transfected alone, the NR2A^{Trunc} was expressed at a significantly lower level than NR2A^{WT}. However, since native receptors are known to be co-assembled from both NR1 and NR2 subunits (Sheng *et al.*, 1994; Blahos and Wenthold, 1996; Chazot and Stephenson, 1997a; Luo *et al.*, 1997), it is more important that NR2A^{Trunc} was expressed to the same level as NR2A^{WT} when it was co-expressed with NR1-1a. Therefore, the NR2A^{Trunc} subunit behaved as the wild-type with respect to the expression level in the NR1/NR2 heteromeric subunit combination. The subsequent characterisation of the expression of truncated NR1-1a/NR2 receptors $\pm$ PSD-95_{c-Myc} revealed that the enhancement of NR2 subunit immunoreactivities was not apparent when either NR1/NR2A^{Trunc} or NR1-1a/NR2B^{Trunc} receptors were co-expressed with PSD-95_{c-Myc}. The increased level of NR2 subunit expression was therefore dependent upon the NR2 C-terminal ESDV motifs.

Analysis of NMDA receptor expression by radioligand binding

The parameters for [³H]MDL 105,519 binding to both NR1-1a/NR2A and NR1-1a/NR2B receptors were comparable in the presence and absence of PSD-95_{c-Myc} (Table 3.2). Because [³H]MDL 105,519 binds with equal high affinity to both assembled and unassembled NR1-1a subunits (Chazot *et al.*, 1998), this is in accord with the quantitative immunoblotting results, *i.e.* no change in the expression level of NR1-1a with or without PSD-95_{c-Myc}.

Saturation [³H]MK801 binding assayed in the presence of 10 μ M L-glutamate and 30 μ M glycine revealed an approximate three-fold increase in the B_{max} value for NR1-1a/NR2A receptors expressed in the presence of PSD-95_{c-Myc} (Table 3.2). Since [³H]MK801 binds with high affinity only to assembled NR1/NR2 receptors (Laurie and Seeburg, 1994), the increase in the number of binding sites showed that PSD-95_{c-Myc} enhanced the level of assembled NMDA receptor. Both PSD-95_{c-Myc} and wild-type PSD-95 enhanced [³H]MK801 binding to NR1-1a/NR2A receptors to the same level, showing that the epitope tagged PSD-95 behaved as wild-type PSD-95. When NR1-1a/NR2A^{Trunc} receptors were co-expressed with PSD-95_{c-Myc}, the three-fold increase in the B_{max} value was significantly reduced and approached control values demonstrating that this effect of PSD-95_{c-Myc} was dependent upon the ESDV C-terminal motif of the NR2A subunit.

Saturation [³H]CGP 39653 binding also revealed an approximate 2.5-fold increase in the B_{max} for NR1-1a/NR2A receptors expressed in the presence of PSD-95_{c-Myc}. Because [³H]CGP 39653, like [³H]MK801, is selective for NMDA receptors co-assembled

from NR1 and NR2 subunits (Kendrick *et al.*, 1996), this is in agreement with the 3-fold increase in the number of [³H]MK801 binding sites detected in HEK 293 cells transfected with NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc}.

Differences in the expression of total NR1-1a receptor subunits compared to the level of NR1-1a in assembled heteromeric NR1-1a/NR2 receptors

The PSD-95_{c-Myc}-mediated increase in the number of [³H]MK801 binding sites is in agreement with the approximate three-fold increase in the intensity of NR2 subunit immunoreactivities. One would predict however, that since PSD-95_{c-Myc} enhanced the expression of assembled NMDA receptors, then a parallel increase in the expression of NR1-1a should be observed. This raises the question of why the level of NR1-1a subunit expression as determined by both quantitative immunoblotting and [³H]MDL 105,519 radioligand binding was unchanged. A possible explanation for this disparity is that in HEK 293 cells transfected with NR1-1a/NR2A, a significant proportion of the expressed NR1-1a subunits exist as an intracellular pool of unassembled subunits (Chazot and Stephenson, 1997b). The presence of unassembled NR1-1a subunits in transfected HEK 293 cells is highlighted by the comparative B_{max} values for [³H]MDL 105,519 and [³H]MK801 binding to HEK 293 cells transfected with NR1-1a/NR2A receptors. The B_{max} for [³H]MDL 105,519, which binds to both unassembled and assembled NR1-1a subunits, was approximately 12-fold greater than that for [³H]MK801, which only binds to assembled NR1-1a/NR2A receptors. Assuming that there are at least two NR1 subunits corresponding to two [³H]MDL 105,519 binding sites per assembled receptor as has been suggested by a number of groups (Behe *et al.*, 1995; Premkumar and Auerbach 1997; Laube *et al.*, 1998), it follows that unassembled NR1-1a subunits outnumber assembled NR1-1a subunits by ~6-fold. The analysis of HEK 293 cell homogenates by quantitative immunoblotting resolves only changes in total cellular protein and does not differentiate between either assembled and unassembled receptor subunits or intracellular and cell surface receptors. Thus, the apparent lack of effect of PSD-95_{c-Myc} on NR1-1a immunoreactivities may be a result of NR1-1a subunits being redistributed from an unassembled pool to the assembled receptor complexes with no change in the absolute level of total NR1-1a subunits. It has been suggested that the availability of the NR2 subunit is the rate-limiting factor in the expression of functional NMDA receptors (Bessho *et al.*, 1994; Zhong *et al.*, 1995). Therefore, if the NR2 subunit is stabilised by PSD-95_{c-Myc}, then the pool of unassembled NR1-1a subunits may be accessed resulting in an increase in the number of assembled

receptors. Alternatively, if the number of NR1-1a subunits in assembled receptors is small in proportion to the number of unassembled NR1-1a subunits, then even a modest increase in the level of assembled or cell surface NR1-1a would be masked by the level of total NR1-1a immunoreactivity. A small proportion of NR2 subunits may also exist in an unassembled state. However, the co-expression of PSD-95_{c-Myc} with NR2A alone also enhanced the expression of NR2A showing that PSD-95_{c-Myc} enhances the level of NR2A in both assembled and unassembled states. The fact that PSD-95_{c-Myc} enhances the level of the unassembled NR2A subunit also suggests that PSD-95_{c-Myc} and NR2A interact at an intracellular location since NR2A expressed alone in HEK 293 cells is not targeted to the cell surface (McIlhinney *et al.*, 1996; McIlhinney *et al.*, 1998).

Mechanisms for the enhanced expression of NMDA receptors in the presence of PSD-95

A number of studies have reported an effect, similar to the one described here, of PSD-95 on the expression of its other associated ion channels. For example, Yamada *et al.* (1999) demonstrated by quantitative immunoblotting that PSD-95 induced an increase in the expression of both NR1 and NR2B subunits when co-expressed in *Xenopus* oocytes. Thomas and Westbrook (1999) reported an enhanced cell surface expression of NR1-1a/NR2A receptors in the presence of PSD-95 as determined by an increase in the peak current amplitude of NMDA receptor-mediated currents. Further, Horio *et al.* (1997) measured a 2-fold increase in K⁺ channel-mediated currents in HEK 293 cells transfected with the Kir4.1 K⁺ channel in the presence of PSD-95, again suggesting a PSD-95-induced increase in the cell surface expression of Kir4.1 channels. The results presented here therefore agree with those of these three groups. Unfortunately, neither the immunoblot analyses nor the [³H]MK801 radioligand binding assays differentiated between the expression of NMDA receptors at the cell surface and at intracellular sites. However, McIlhinney *et al.* (1996, 1998) showed that assembled NR1-1a/NR2A receptors were expressed at the cell surface of HEK 293 cells whereas the unassembled subunits remained in intracellular compartments. This suggests that receptors constituting [³H]MK801 binding sites, *i.e.* assembled NR1-1a/NR2A receptors, are expressed at the cell surface and thus, PSD-95 probably enhances the cell surface expression of NMDA receptors. Experiments to quantify the effect of PSD-95 on the cell surface expression of NR1-1a/NR2A receptors by cell surface enzyme-linked immunosorbent (ELISA) assay are currently in progress (Freeman *et al.*, *In Press*).

A number of recent studies have shed light on the possible mechanisms for the enhanced cell surface expression of NMDA receptors observed in the presence of PSD-95. For example, the co-expression of the K⁺ channel, Kv1.4, with PSD-95 slowed the internalization rate of the cell surface K⁺ channels (Jugloff *et al.*, 2000). Furthermore, a recent study identified a tyrosine motif, YXXL, in the NR2B subunit C-terminus that stimulates the internalization of cell surface NMDA receptors in primary cultures of cortical neurons (Roche *et al.*, 2000). It was proposed that the binding of PSD-95 to the NR2B subunit masks this internalization sequence resulting in the enhanced cell surface expression of the receptor. Since a similar internalization motif, YXXM, is present in the NR2A subunit, it is possible that the same mechanism may occur in HEK 293 cells such that the association of PSD-95 with the NR2A subunit reduces the internalization rate of NMDA receptors resulting in the accumulation of receptors at the cell surface. Internalization of NMDA receptors by endocytosis in the absence of PSD-95 may lead to their proteolytic degradation. A reduction in the rate of receptor internalization in the presence of PSD-95 would therefore lead to an increase in the overall number of assembled NMDA receptors.

PSD-95 has been shown to associate not only with receptors and ion channels but directly and indirectly with a number of cytoskeletal and structural proteins that are present in the post-synaptic densities of mammalian brains. These include neuroligins, CRIPT, GKAP, and SHANK (Irie *et al.*, 1997; Neithammer *et al.*, 1998; Passafaro *et al.*, 1999; Takeuchi *et al.*, 1997; Kim *et al.*, 1997; Naisbitt *et al.*, 1997; Naisbitt *et al.*, 1999). Although these particular PSD-95-associated proteins may not be present in HEK 293 cells, the PDZ domains of PSD-95 have multiple and diverse binding partners (reviewed in Nagano *et al.*, 1998). Therefore, the enhanced expression of NMDA receptors in the presence of PSD-95 may occur via the association of PSD-95 with other intracellular PDZ binding proteins. These may stabilize the receptor in the plasma membrane or again, prevent the proteolytic degradation of NMDA receptors via an endocytic pathway.

It is interesting that in the study by McIlhinney *et al.* (1998), the majority of NR2A subunits expressed at the cell surface of HEK 293 cells transfected with NR1-1a/NR2A receptors, were C-terminally truncated. Since the antibody used in this thesis to detect the NR2A subunit was raised against a peptide located approximately 50 amino acids upstream of the C-terminus, truncated NR2A subunits would not have been detected by immunoblotting. It is possible that PSD-95 protects the NR2A subunit from this C-terminal truncation resulting in an increase in the proportion of NR2A subunits

containing the anti-NR2A (1,381-1,394) epitope. The PSD-95-induced increase in the intensity of NR2A subunit immunoreactivities observed by immunoblotting using the anti-NR2A (1,381-1,394) antibodies may not therefore, have been due to an enhanced expression of the NR2A subunit. Instead, PSD-95 may just prevent the truncation of the epitope for anti-NR2A (1,381-1,394) antibodies. An N-terminally directed NR2A antibody was not available to investigate this possibility. However, given that PSD-95 also enhanced the level of [³H]MK801 binding sites, it is probable that the increase in the intensity of NR2A immunoreactivity does reflect an enhanced expression of the NR2A subunit rather than an inhibition of the C-terminal truncation. It is notable that using an antibody directed against the N-terminus of the NR2B subunit (Figure 3.21), a C-terminally truncated NR2B subunit was not detected in transfected HEK 293 cells. The C-terminal truncation of NR2A reported by McIlhinney *et al.* (1998) may therefore be specific to the NR2A subunit.

Insights into the function of PSD-95 *in vivo*

The enhanced expression of cloned NMDA receptors by PSD-95 may have important consequences for the control of receptor number at post-synaptic sites *in vivo*. The magnitude of the NMDA receptor-mediated component of the excitatory post-synaptic current (EPSC) is dependent upon the number of receptors at particular synaptic sites. Therefore, the precise number and density of NMDA receptors located in the plasma membrane of post-synaptic excitatory neurons may be crucial for the NMDA receptor-dependent regulation of synaptic transmission. Indeed, the over-expression of NR2B in genetically altered mice results in an enhancement of learning and memory (Tang *et al.*, 1999).

It is not known what proportion of NMDA receptors are associated with PSD-95 or whether this association is regulated. However, it is becoming increasingly clear that the post-synaptic density is a dynamic macromolecular structure. A number of signalling proteins, for example CaMK-II, and indeed AMPA receptors appear to be continuously translocated into and out of the PSD (reviewed in Meyer and Shen, 2000). PSD-95-clusters in cultured hippocampal neurons also appear to be continually remodelled and the clustering is regulated by synaptic activity (Okabe *et al.*, 1999b). All this evidence points towards the association between PSD-95 and NMDA receptors as being highly regulated raising the possibility that an altered interaction of NMDA receptors with PSD-95 may reduce the stability of NMDA receptors in the post-synaptic membrane and have profound consequences on neuronal function. In the PSD-95-knockout mouse however, the

morphology of glutamatergic synapses was not altered (Migaud *et al.*, 1998). Furthermore, the NMDA receptor-mediated component of the EPSC was unchanged suggesting that the number of NMDA receptors residing in the post-synaptic membrane was not reduced. The association of PSD-95 with the receptor may not therefore be necessary for the control of the NMDA receptor cell surface expression *in vivo*. However, other known NMDA receptor-associated proteins were present in the PSDs of the PSD-95-knockout mouse including PSD-93 and SAP102, and these may have compensated for the lack of PSD-95.

In contrast to the PSD-95-knockout mouse, the punctate clustering and synaptic localization of NR2A was inhibited in genetically altered mice expressed a C-terminally truncated NR2A subunit (NR2A^{ΔC}) (Steigerwald *et al.*, 2000). The number of NR2A subunits enriched in the isolated PSD fraction was significantly reduced compared to wild-type mice whereas the number of extra-synaptic receptors was not altered. A 43 % decrease in the amplitude of action potential-evoked EPSCs (eEPSCs) in NR2A^{ΔC} mice also demonstrated a decrease in the number of synaptic NR1/NR2A receptors. Furthermore, the overall expression of the NR2A subunit was reduced in NR2A^{ΔC} mice which is consistent with a decreased number of NR2A subunits rather than a redistribution of NR2A to non-synaptic areas. The decrease in the overall expression of the NR2A subunit in the NR2A^{ΔC} mutant mice suggests that indeed an association with PSD-95 may be important for the regulation of receptor number at the synapse. The apparent lack of effect of the C-terminal truncation on the number of extra-synaptic receptors, which would not be associated with PSD-95 in wild-type mice, clearly demonstrates that the reduced expression of NR2A at the synapses of NR2A^{ΔC} mice is due to a lack of an interaction between truncated NR2A and proteins of the PSD. These effects of the C-terminal truncation in NR2A^{ΔC} mice yield more insights into the importance of the interaction of NMDA receptors with PSD-95-like proteins compared to the PSD-95-knockout since compensatory contributions of other PSD-95-like proteins in the PSD-95-knockout would presumably not occur in the NR2A^{ΔC} mice. The disadvantage however, is that since a C-terminally truncated NR2A subunit would not interact with any of the PSD-95-like proteins, the consequences of the C-terminal truncation cannot then be attributed to a lack of an interaction with any particular PSD protein. It would be interesting to compare the turnover rates of the cell surface NR2A subunits in neurons from wild-type mice and NR2A^{ΔC} mice. This may provide further insights into the role of PSD-95-like proteins in the regulation of the expression of NMDA receptors at synaptic sites and may clarify the relevance of the effect of PSD-95 on cloned receptors to the properties of native NMDA receptors.

The ability of PSD-95 to enhance the number of NMDA receptors expressed in HEK 293 cells has implications for the properties of extra-synaptic NMDA receptors that have been identified in some neurons of the CNS (Baude *et al.* 1994; Li *et al.*, 1998; Tovar and Westbrook, 1999). The lack of an interaction between extra-synaptic NMDA receptors and proteins of the PSD, especially PSD-95, may result in altered stabilities of these receptors in the plasma membrane. Indeed, as shown by Steigerwald *et al.* (2000), the number of extra-synaptic NR2A subunits expressed in NR2A^{ΔC} mice was not altered when compared to wild-type mice indicating that the expression level of extra-synaptic receptors in the membrane is not subject to regulation by the PSD proteins that are associated with C-termini of synaptic receptors. Although the function of these extra-synaptic receptors is unknown, they may display important functional differences due to the altered interaction with PSD-95.

The relevance of the *in vitro* observations in this thesis to native NMDA receptors is of course speculative. In fact, the ability of PSD-95 to enhance the number of NMDA receptors expressed in transfected HEK 293 cells may not be related to its function *in vivo*. The expression of recombinant receptors in non-neuronal cell lines such as HEK 293 cells is a useful system to study the properties of the NMDA receptor in isolation and in the presence of PSD-95. However, the disadvantage is that in the absence of many other receptor-associated proteins, the cell surface targeting, internalization and degradation of cloned NMDA receptors may not be truly representative of native NMDA receptors. The effects of PSD-95 on the properties of the NMDA receptor may possibly occur only in these non-neuronal expression systems. Despite these disadvantages, the control of receptor turnover at the plasma membrane appears to be a common function of other known receptor-associated proteins expressed in both heterologous cells and cultured neurons indicating that the effect of PSD-95 on the expression of NMDA receptors may indeed relate to its function *in vivo*. For example, co-expression of the nAChR-interacting protein, rapsyn, with nAChRs in cultured myotubes slows the turnover of nAChRs by approximately two-fold (Wang *et al.*, 1999). The co-expression in HEK 293 cells of either Homer 1a or Homer 1c with mGluR1 α also increases the amount of cell surface receptor by reducing the rate of receptor internalization (Ciruela *et al.*, 1999; Ciruela *et al.*, 2000). In contrast, the mGluR-interacting protein, Homer 1b, inhibits the surface expression of mGluR5 in heterologous cells whereas when expressed alone, mGluR5 successfully travels through the secretory pathway to the plasma membrane (Roche *et al.*, 1999). Interestingly, the expression of AMPA receptors in neurons also appears to be highly dynamic with

respect to their localization at post-synaptic sites and their metabolic stability in the plasma membrane (Mammen *et al.*, 1997; Lissin *et al.*, 1999). A striking effect of an interacting protein on the surface expression of AMPA receptors was recently demonstrated by Noel *et al.* (1999). Inhibition of the interaction between GluR2 and N-ethylmaleimide-sensitive fusion protein (NSF) reduced the surface expression of AMPA receptors and reduced synaptic transmission in cultured hippocampal neurons (Noel *et al.*, 1999; Nishimune *et al.*, 1998). Furthermore, blockade of the NSF/AMPA receptor interaction prevented the induction of LTD suggesting that the control of receptor number in the post-synaptic membrane by NSF is important for the induction of LTP and LTD (Luthi *et al.*, 1999).

4.2.2 The splice form of the NR1 subunit regulates the expression of NMDA receptors

Enhanced expression of assembled NMDA receptors containing NR1-2a or NR1-4b splice variants

The analysis of HEK 293 cell homogenates transfected with NR1-1a/NR2A, NR1-2a/NR2A or NR1-4b/NR2A by quantitative immunoblotting revealed that the splice form of the NR1 subunit influenced the level of total NR1 subunit expression and the number of assembled NR1/NR2A receptors in HEK 293 cells. The results are summarised in Table 4.2.

Receptor preparation	NR1 immunoreactivity	NR2A immunoreactivity	[³ H]MK801 binding activity
NR1-2a/NR2A	↓	↔	↑
NR1-4b/NR2A	↓↓	↔	↑↑

Table 4.2 Summary of the effect of the NR1 subunit subtype on the expression of NMDA receptors in HEK293 cells

The expression of NR1-2a/NR2A and NR1-4b/NR2A receptors is shown relative to the level of NR1-1a/NR2A receptor expression. ↓, a relative decrease compared to NR1-1a/NR2A. ↑, a relative increase. ↔, no change.

The expression of total NR1-2a and NR1-4b subunits in NR1/NR2 combinations was decreased by approximately 40 % and 70 % compared to NR1-1a respectively (Figure

3.30). However, [³H]MK801 binding studies revealed that despite the decreased expression of total NR1-2a and NR1-4b, the levels of assembled NR1-2a/NR2A and NR1-4b/NR2A were enhanced by approximately 25 % and 75 % respectively compared to NR1-1a/NR2A receptors (Figure 3.31). This enhanced assembly of both NR1-2a/NR2A and NR1-4b/NR2A receptors must be due to the presence or the absence of alternatively spliced exons of the NR1 gene, *i.e.* the absence of the C1 exon in the NR1-2a subunit compared to NR1-1a and the combined effects of the absence of the C1 and the presence of the N1 and C2' exons in NR1-4b.

The increase in the number of assembled NR1-4b/NR2A receptors compared to NR1-1a/NR2A receptors despite the decreased expression of total NR1-4b compared to NR1-1a may be related to the existence of an unassembled pool of NR1-1a subunits in NR1-1a/NR2A transfected HEK 293 cells (Chazot and Stephenson, 1997b). If the level of assembled NR1-4b/NR2A is ~75 % greater than the assembled NR1-1a/NR2A receptors even though the expression of total NR1-4b subunit is decreased by ~70 % compared to total NR1-1a, it follows that at least ~80 % of NR1-1a is unassembled. It is evident that at least for NR1-4b therefore, a substantially greater proportion of the NR1 subunits are assembled with NR2A subunits compared to NR1-1a. It is not so clear cut for NR1-2a. The level of NR1-2a was significantly decreased compared to NR1-1a but in contrast to NR1-4b/NR2A, the level of assembled NR1-2a/NR2A was not significantly greater than NR1-1a/NR2A. Therefore, contributions of either the N1 exon or the C2 exon to the control of receptor assembly and expression appear to be more significant compared to the C1 exon.

Does the NR1 subunit splice form regulate cell surface expression of NR1/NR2A receptors?

A recent study by Okabe *et al.*, (1999a) reported that alternative splicing of the NR1 subunit altered the cell surface expression of NR1/NR2B receptors in transfected mouse fibroblasts. The expression of all the NR1 subunit splice variants was quantified by immunofluorescence and revealed firstly, that in contrast to the results in this thesis, the total cellular expression, *i.e.* surface and intracellular expression, of each splice variant was equal. However, the cell surface expression of NR1-2b/NR2B and NR1-4b/NR2B was enhanced by approximately 100 % and 200 % respectively, compared to NR1-1b/NR2B receptors. In addition, the NR1 N1 exon did not influence the cell surface expression of NR1/NR2B receptors. A significant difference in the cell surface expression of the NR1

splice variants was also observed when the NR1 subunits were expressed in the absence of NR2B. The NR1-1b subunit was totally absent from the cell surface whereas both the NR1-2b and NR1-4b were expressed at the cell surface. This suggests that the enhanced levels of assembled NR1-4b/NR2A receptor observed in this thesis may be due to an enhanced cell surface expression.

Mechanisms for the enhanced expression of assembled NR1-4b/NR2A receptors

A number of recent studies have attempted to map the various amino acid sequences located in the NR1-1a subunit that are responsible for the retention of NR1-1a as unassembled subunit inside the cell. Indeed, the presence of an ER retention signal located in the C1 exon of the NR1-1 and NR1-3 splice variants was recently reported (Standley *et al.*, 2000; Holmes *et al.*, 2000). Standley *et al.* (2000) propose that the NR1-2a and NR1-4b subunits are expressed at the cell surface of transfected neurons because they lack this retention signal. It is therefore possible that in HEK 293 cells, the retention of NR1-1a in the ER reduces the efficiency with which NR1-1a/NR2A receptors are assembled and transported to the cell surface, resulting in a decreased level of assembled NR1-1a/NR2A receptors compared to NR1-2a/NR2a and NR1-4b/NR2A. This does not however explain why the total cellular expression of NR1-1a is greater than both NR1-2a and NR1-4b. It may be that the unassembled NR1-1a subunits retained in the ER are not subjected to proteolytic degradation via an exocytic/endocytic pathway and therefore accumulate as an unassembled pool in the ER. In contrast, the NR1-2a and NR1-4b subunits may be transported to the cell surface more efficiently where they remain until they are degraded via an endocytic pathway. This continual recycling and degradation of the NR1-2a and NR1-4b subunits may result in a decreased expression of total NR1 subunit compared to NR1-1a despite the increase in the number of assembled receptors.

The association of other NMDA receptor-associated proteins with the NMDA receptor in HEK 293 cells may also regulate the assembly and stability of NR1-1a/NR2A receptors. The C1 exon is known to mediate an interaction between NMDA receptors and both the protein yotiao and the cytoskeletal protein NF-L (Ehlers *et al.*, 1998; Lin *et al.*, 1998). The differential association of the NR1 splice variants with other associated proteins that might be present endogenously in HEK 293 cells may therefore contribute to variations in trafficking, assembly and stabilization of cloned NMDA receptors.

4.2.3 Does the NR1 subunit splice form regulate the enhanced expression of NR1/NR2A receptors by PSD-95?

The effect of the co-expression of PSD-95_{c-Myc} on the expression of NR1/NR2A receptors was found to be regulated by the NR1 subunit splice form. The results are summarised in Table 4.3.

Receptor preparation	NR1 immunoreactivity	NR2A immunoreactivity	[³ H]MK801 B _{max}
NR1-1a/NR2A	↔	↑↑↑	↑↑↑
NR1-2a/NR2A	↑	↑↑↑	↑↑
NR1-4b/NR2A	↑↑	↑↑↑	↑

Table 4.3 Summary of the effect of the NR1 subunit subtype on the PSD-95- enhanced expression of NMDA receptors in HEK 293 cells

↑, an increase in immunoreactive intensities or [³H]MK801 B_{max} in the presence of PSD-95 compared to experiments performed in the absence of PSD-95. ↔, no effect of PSD-95.

With respect to the effect of PSD-95_{c-Myc} on the level of NR2A expression, the NR1 splice form did not influence the three-fold increase in NR2A subunit immunoreactivities induced by PSD-95_{c-Myc} (Figure 3.30). However, in the presence of PSD-95_{c-Myc}, the expression level of the NR1-4b subunit in an NR1/NR2A combination was significantly enhanced (1.8-fold). This is in contrast to the apparent lack of effect of PSD-95 on the expression of NR1-1a. A small, *i.e.* 1.2-fold, increase in the level of NR1-2a expression was observed but the immunoblotting procedure was not sensitive enough to resolve this to a level of significance. With respect to the effect of PSD-95_{c-Myc} on the level of assembled NR1/NR2A receptors as determined by [³H]MK801 binding, the number of both NR1-2a/NR2A and NR1-4b/NR2A receptors was enhanced by the co-expression of PSD-95_{c-Myc} but the magnitude of this enhancement was lower than that observed for NR1-1a/NR2A, *i.e.* 2.0-fold and 1.4-fold respectively compared to 3.1-fold (Figure 3.31).

Why might the co-expression of PSD-95_{c-Myc} with NR1-4b/NR2A receptors result in an increase in the total expression level of NR1-4b but not NR1-1a? It was hypothesised earlier that the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors did not result

in an increase in the NR1-1a subunit immunoreactivity even though the level of assembled NR1-1a/NR2A was increased by PSD-95_{c-Myc} because the pool of unassembled NR1-1a masked any changes in the level of total NR1-1a detected by immunoblotting. However, as discussed in section 4.2.2, a greater proportion of the NR1-4b subunit must exist as assembled NR1/NR2A receptor compared to NR1-1a. The increase in the number of assembled NR1-4b/NR2A receptors induced by PSD-95_{c-Myc} would therefore be paralleled by an increase in the intensity of both NR1-4b and NR2A subunit immunoreactivities corresponding to total NR1-4b and NR2A. With respect to the NR1-2a splice variant, there was only a slight increase in the intensity of the NR1-2a subunit immunoreactivity detected in the presence of PSD-95_{c-Myc}. Therefore, a significant proportion of the NR1-2a subunit must still exist as unassembled receptor such that in the presence of PSD-95_{c-Myc}, the increase in the level of NR1-2a expression would be masked by the presence of unassembled NR1-2a subunits.

It cannot be ruled out that an association between PSD-95 and NR1-4b may contribute to the observed effects of PSD-95 on the expression of NR1-4b/NR2A receptors. The NR1-4b subunit has a consensus tSXV motif at its C-terminus and the co-immunoprecipitation of NR1-4b with PSD-95 from transfected HEK 293 cells was recently demonstrated (Standley *et al.*, 2000). This is in agreement with the association of NR1-4b and PSD-95 in a yeast-two hybrid gene-reporter system reported by Kornau *et al.* (1995), but in contrast to Bassand *et al.* (1999) who, using a similar protein-protein interaction assay, found no association. If PSD-95 does interact with NR1-4b in HEK 293 cells, then the effect of PSD-95 on the enhancement of NR1-4b subunit expression may have been observed due to the association of PSD-95 with both assembled and unassembled NR1-4b receptor subunits.

The second question to address is why the PSD-95_{c-Myc}-induced increase in the number of assembled NR1-4b/NR2A and NR1-2a/NR2A receptors was less than the increase in assembled NR1-1a/NR2A receptors? A possible explanation for this is that the number of assembled NR1-4b/NR2A and NR1-2a/NR2A receptors detected in the absence of PSD-95_{c-Myc} was already greater than that of NR1-1a/NR2A receptors. Presumably, there is a limited number of receptors that the transfected cell can assemble and express on the cell surface. If the NR1-4b/NR2A and NR1-2a/NR2A receptors are already expressed more efficiently at the cell surface, then the co-expression of PSD-95_{c-Myc} may not result in such a significant percentage increase in the number of assembled NR1-4b/NR2A and NR1-2a/NR2A receptors.

In summary, the difference in the magnitude of the PSD-95_{c-Myc}-induced increase in the level of assembled NR1/NR2A receptors between the alternatively spliced NR1 subunits appears to be due to differences in the initial level of assembled NR1/NR2A receptor in the absence of PSD-95_{c-Myc}. In addition, the altered effect of PSD-95_{c-Myc} on the total expression of NR1 subunits can be explained by the splice-dependent differences in the relative contribution of unassembled NR1 subunits to the total NR1 immunoreactivity. It is clear however, that whatever factor is responsible for the decreased expression of NR1-1a/NR2A receptors compared to NR1-2a/NR2A and NR1-4b/NR2A receptors, the co-expression of PSD-95_{c-Myc} overcomes this inhibitory effect. Perhaps by interacting with the NR2A subunit of the NR1-1a/NR2A receptor complex, PSD-95_{c-Myc} masks the effect of an ER retention signal located in the C1 exon the NR1-1a subunit.

Does alternative splicing regulate the expression of NMDA receptors *in vivo*?

It is not clear whether the subcellular localization and expression of NMDA receptors in transfected mammalian cells is representative of native NMDA receptors. However, it is possible that the effect of the NR1 subunit splice form on the expression of NR1/NR2A receptors in HEK 293 cells may be relevant to the assembly and cell surface expression of NMDA receptors in neuronal cells. It is thought that the majority of native NR1-4b subunits are assembled to form functional NMDA receptors in mammalian brains whereas C2 exon containing NR1-1a receptors form a substantial pool of unassembled NR1 subunits (Chazot and Stephenson, 1997b; Hall and Soderling, 1997; Huh and Wenthold, 1999). In this respect, the relative distribution of NR1-1a and NR1-4b subunits in HEK 293 cells agrees with observations *in vivo*. The function of the pool of unassembled NR1-1a subunits found *in vivo* is not known. However, alternative splicing of the NR1 subunit may be a means of regulating the level of cell surface NR1/NR2A receptors.

The regulation of expression, assembly, and turnover of NMDA receptors expressed in HEK 293 cells and in neurons is likely to be highly complex. The resulting level of NMDA receptors expressed in HEK 293 cells in the presence and absence of PSD-95 appears to be governed by the combination of multiple factors. The expression of assembled NMDA receptors in the absence of PSD-95 is probably dependent upon the efficiency with which receptors are assembled in the ER, on whether individual subunits are retained in the ER, on the efficiency with which they are transported to the cell surface, on their susceptibility to proteolytic degradation, and on the rate of receptor internalization. The alternative splicing of the NR1 subunit appears to be critical in the regulation of these

pathways and may determine the final density of NMDA receptors at the post-synaptic membrane. This is in contrast to the suggestion that the expression of NR2A is the rate-limiting factor for the assembly of functional NMDA receptors in transfected cells and neurons (Zhong *et al.*, 1995). These results suggest that both the expression of NR2A and the splice-dependent trafficking of NR1 subunits contribute to the assembly of NR1/NR2A receptors. The association of PSD-95 with the NR2 subunit or C2'-containing NR1 subunits may also alter the efficiency with which NMDA receptors are processed during this exocytic/endocytic pathway resulting in an increase in the level of assembled NMDA receptor. As described in section 4.2.1, the control of receptor number in the post-synaptic membrane is likely to be critical for the regulation of neuronal function. Therefore, alternative splicing of the NR1 subunit may be another mechanism responsible for the modulation of synaptic transmission.

4.3 Does PSD-95 alter the post-translational modification or the proteolytic degradation of NR1-1a and NR2A subunits expressed in HEK 293 cells?

It was observed that anti-NR2A (1,381-1,394) antibodies and anti-NR1-1a (911-920) antibodies sometimes detected doublets in cells transfected with NR1-1a/NR2A (Figures 3.5, 3.22 and 3.30). The additional lower molecular weight species detected with anti-NR1 (911-920) antibodies may be either a non-glycosylated form of the NR1-1a subunit or a proteolytic fragment of NR1-1a. Chazot *et al.* (1992) previously studied the N-glycosylation of NR1-1a subunits in transfected HEK 293 cells using N-glycanase. Treatment of transfected cell membranes with N-glycanase resulted in a decrease of the NR1 subunit with $M_r = 120$ kDa and a concomitant increase in the $M_r = 97$ kDa immunoreactive species suggesting that the lower molecular weight species was the non-glycosylated form of NR1-1a. In immunoblots where the additional lower molecular weight band was detected, in the presence of PSD-95_{c-Myc}, only the higher molecular weight band was present. The co-expression of PSD-95 with NR1-1a/NR2A receptors may either protect the NR1-1a subunit from proteolytic degradation or it may enhance the glycosylation of NR1-1a. Interestingly, an alternative splice form of PSD-95, PSD-95B, has been shown to inhibit the glycosylation of Kv1.4 channels (Bunn *et al.*, 2000). PSD-95B, which lacks the two N-terminal cysteine residues, C3 and C5, clusters Kv1.4 channels at intracellular sites, whereas PSD-95 clusters Kv1.4 channels at the cell surface. As a consequence, the co-expression of PSD-95B with Kv1.4 channels resulted in the

appearance of immature non-glycosylated Kv1.4 channels in transfected cells. In the presence of PSD-95, only the mature glycosylated form was detected. The most likely explanation for the results presented in this study is that the NR1-1a, $M_r = 97$ kDa, immunoreactive species is non-glycosylated NR1-1a subunit and that the co-expression of PSD-95 enhances the glycosylation of NR1-1a. PSD-95 may therefore be involved in the trafficking of NMDA receptors through the ER and the Golgi apparatus to the cell surface, resulting in the enhanced glycosylation of NR1-1a subunits.

The lower molecular weight band of $M_r = 175$ kDa detected with anti-NR2A (1,381-1,394) antibodies may also be a non-glycosylated form of the NR2A protein. The glycosylation of NR2A subunits has previously been investigated using tunicamycin (TM) which inhibits the synthesis of asparagine-linked oligosaccharides (Chazot *et al.*, 1995). Culturing of the cells in the presence of TM resulted in a decrease in the mature NR2A subunit, $M_r = 180$ kDa, and an increase in the $M_r = 165$ kDa, non-glycosylated NR2A subunit. The M_r of the lower molecular weight species shown in Figures 3.22 and 3.30 is slightly larger than that of the non-glycosylated NR2A subunit described by Chazot *et al.* (1995), and alternatively it may be a degraded form of NR2A. The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors may therefore protect the NR2A subunit from proteolytic degradation or it may enhance the glycosylation of NR2A.

4.4 THE EFFECT OF PSD-95 ON THE MOLECULAR PHARMACOLOGICAL PROPERTIES OF THE NMDA RECEPTOR

Initial studies revealed that PSD-95_{c-Myc} did not change the parameters for [³H]MDL 105,519 binding to NR1-1/NR2A receptors. The K_{DS} values for [³H]MDL 105,519 binding to NR1-1a/NR2A receptors were in agreement with the corresponding parameters determined previously for [³H]MDL 105,519 binding to rat forebrain membranes and recombinant NR1-1a/NR2A receptors (Baron *et al.*, 1996; Chazot *et al.*, 1998). In contrast, PSD-95 significantly altered the parameters for [³H]MK801 binding to NR1-1a/NR2A receptors. The coexpression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors resulted in a decrease in the affinity for MK801 determined in the presence of 10 μ M L-glutamate and 30 μ M glycine. Subsequently, it was found that PSD-95 decreased the sensitivity of [³H]MK801 binding to allosteric modulation by both L-glutamate and glycine, as determined by the L-glutamate- and glycine-dependent enhancement of [³H]MK801 binding. The reduced sensitivity to L-glutamate explained the apparent decrease in affinity

for MK801 binding because in the presence of saturating concentrations of L-glutamate, *i.e.* 1 mM, the K_i values for MK801 binding to NR1-1a/NR2A receptors were identical in the presence and absence of PSD-95_{c-Myc}. Further radioligand binding analyses revealed that PSD-95_{c-Myc} did not change the affinity of NR1-1a/NR2A receptors for either L-glutamate or glycine suggesting that PSD-95_{c-Myc} may inhibit NMDA receptor channel gating, *i.e.* the isomerization of the agonist-bound receptor to an active state, rather than inhibiting agonist binding *per se*. A possible theory for the mechanism of action for the PSD-95-mediated decrease in sensitivity to L-glutamate was that PSD-95_{c-Myc} may change the phosphorylation state of the NR1 subunit via sites located in the alternatively spliced exons. Therefore, the effect of the NR1 subunit splice form on the enhancement of [³H]MK801 binding by L-glutamate $\pm$ PSD-95_{c-Myc} was investigated. It was found that the decrease in L-glutamate sensitivity was independent of the N1, C1 and C2 exons suggesting that if PSD-95_{c-Myc} mediates its effects via a change in the phosphorylation state of the NMDA receptor, it does not occur via these NR1 subunit sequences.

The effect of PSD-95 on the radioligand binding parameters for NR1-1a/NR2A receptors is summarised in Table 4.4 overleaf. These results are discussed in detail in the following sections with particular reference to the properties of cloned NMDA receptors $\pm$ PSD-95 compared to native receptors, the observed effects of PSD-95 compared to recent published studies and the relevance of these effects *in vivo*.

Ligand	Parameter	Assay conditions	Effect of PSD-95
MK801	K _I	+ 10 μ M L-glutamate	↑ ↑ ↑
MK801	K _I	+ 1 mM L-glutamate	↔
[³ H]MK801	K _D	+ 10 μ M L-glutamate	↑ ↑ ↑
[³ H]MK801	K _D	+ 1 mM L-glutamate	↑
Glutamate	EC ₅₀	[³ H]MK801 enhancement	↑ ↑ ↑
Glycine	EC ₅₀	[³ H]MK801 enhancement	↑ ↑ ↑
[³ H]CGP 39653	K _D	Standard	↔
[³ H]MDL105,519	K _D	Standard	↔
L-glutamate	K _I	Displacement of [³ H]CGP 39653	↔
Glycine	K _I	Displacement of [³ H]MDL105,519	↔

Table 4.4 A summary of the effects of PSD-95 on the radioligand binding properties of NR1-1a/NR2A receptors expressed in HEK 293 cells

The effect of PSD-95 on the radioligand binding properties of NR1-1a/NR2A was determined using a number of different radioligands and via a variety of binding assays. ↑, a PSD-95-induced increase in the appropriate parameter. ↔, no change.

4.4.1 PSD-95_{c-Myc} reduces the affinity of NR1-1a/NR2A receptors for MK801

MK801 binding to both NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} and native rat forebrain receptors was best fit to a single binding site model consistent with a single MK801 binding site per receptor complex. Co-expression of PSD-95_{c-Myc} resulted in an approximate 3-fold increase in the K_D for direct [³H]MK801 binding and a 5-fold increase in the apparent K_I determined by displacement with MK801 (Table 3.2). The K_D and K_I values determined for MK801 binding to NR1-1a/NR2A receptors which were 14 ± 4 nM and 12 ± 7 nM respectively are consistent with results previously obtained in our laboratory and with those reported by other groups (Chazot *et al.*, 1994; Lynch *et al.*, 1995). The apparent K_I = 13 ± 8 nM for MK801 binding to rat forebrain membranes is also consistent with previously published studies (*e.g.* Wong *et al.*, 1988; Chazot *et al.*, 1994; Porter and Greenamyre, 1995).

The reduced affinity of NR1-1a/NR2A receptors for MK801 in the presence of

PSD-95_{c-Myc} was subsequently shown to be due to a reduced sensitivity of [³H]MK801 binding to allosteric modulation by L-glutamate. As shown in Figure 3.11, a concentration of 10 μ M L-glutamate was saturating for the L-glutamate enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc} but in the presence of PSD-95_{c-Myc}, 10 μ M was only just greater than the EC₅₀ for the enhancement of [³H]MK801 binding. The concentration of L-glutamate (10 μ M) used in the initial MK801 competition assays was therefore saturating for NR1-1a/NR2A receptors but was subsaturating for NR1-1a/NR2A receptor in the presence of PSD-95_{c-Myc}. In contrast, 30 μ M glycine was saturating for NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc}. The decreased sensitivity of NR1-1a/NR2A receptors for glycine was therefore not the probable explanation for the decreased affinity of NR1-1a/NR2A receptors + PSD-95_{c-Myc} for MK801. Since L-glutamate has been shown to increase the affinity of NMDA receptors for [³H]MK801 concentration-dependently (Foster and Wong, 1987; Ransom and Stec, 1988), it was predicted that by increasing the L-glutamate concentration to 1 mM, the affinity of NR1-1a/NR2A receptors in the presence of PSD-95_{c-Myc} for MK801 would be increased and thus be comparable to the affinity of NR1-1a/NR2A receptors expressed alone. Indeed, the K_i values determined for MK801 binding to NR1-1a/NR2A receptors in the presence of 1 mM L-glutamate were not significantly different in the presence and absence of PSD-95_{c-Myc} (Figure 3.16). Furthermore, the displacement curves clearly demonstrated an increase in the affinity of NR1-1a/NR2A receptors + PSD-95_{c-Myc} for MK801 in the presence of 1 mM L-glutamate compared to 10 μ M L-glutamate (Figure 3.15). Both the K_i value was decreased in the presence of 1 mM L-glutamate and the specific binding of [³H]MK801 (10 nM), in the absence of added MK801, to NR1-1a/NR2A/PSD-95_{c-Myc} receptors was enhanced to approximately 1.5 times the specific binding determined in the presence of 10 μ M L-glutamate.

According to the equation for the binding of a ligand to a single site, the *Langmuir* isotherm (shown below), a decrease in the K_D would result in an increase in the level of bound radioligand [RL] if the B_{max} and [L] are constant. Therefore, does the magnitude of the change in specific [³H]MK801 binding to NR1-1a/NR2A/PSD-95_{c-Myc} receptors resulting from an increase in the concentration of L-glutamate correlate with the fold change in K_i observed for MK801 binding?

$$[RL] = \frac{[L] \cdot B_{\max}}{K_D + [L]}$$

[L] = concentration of radioligand

[RL] = concentration of bound radioligand

In a single experiment, for example the one represented in Figure 3.15 C and D, [³H]MK801 binding to NR1-1a/NR2A/PSD-95_{c-Myc} receptors in the presence of either 1 mM L-glutamate or 10 μM L-glutamate was performed on the same transfected cell preparation. The number of NMDA receptors, *i.e.* the B_{max} , is therefore the same in both binding assays. In addition, the concentration of [³H]MK801, *i.e.* [L], used in each displacement assay was identical. According to the above equation, the observed 1.5-fold increase in [RL] would then correlate with an approximate 2-fold decrease in the K_D for [³H]MK801. Therefore, the 5-fold decrease in the apparent K_i observed for MK801 binding to NR1-1a/NR2A/PSD-95_{c-Myc} receptors in the presence of 1 mM L-glutamate compared to 10 μM L-glutamate is probably greater than the true difference. This is probably due to inaccuracies in determining K_i values by homologous competitive binding assays.

The effect of the increased L-glutamate concentration on the affinity of NR1-1a/NR2A receptors ± PSD-95_{c-Myc} for [³H]MK801 was also investigated by saturation radioligand binding. Having shown that in the presence of 1 mM L-glutamate the K_s for MK801 binding to NR1-1a/NR2A receptors were identical in the presence and absence of PSD-95_{c-Myc}, it was predicted that the K_D values, determined by direct [³H]MK801 binding would also be identical ± PSD-95_{c-Myc}. It was found however, that even in the presence of 1 mM L-glutamate, the co-expression of PSD-95_{c-Myc} resulted in a small but significant increase in the K_D for [³H]MK801 binding to NR1-1a/NR2A receptors (Figure 3.23). Despite this difference, the affinity of NR1-1a/NR2A/PSD-95_{c-Myc} receptors for [³H]MK801 was increased in the presence of 1 mM L-glutamate compared to 10 μM L-glutamate. The initial decrease in the affinity of NR1-1a/NR2A receptors for MK801 induced by PSD-95_{c-Myc} in the presence of 10 μM L-glutamate can still therefore be explained by a reduced sensitivity to L-glutamate, but it appears that PSD-95_{c-Myc} may also reduce the affinity of the open channel binding site for [³H]MK801 even under maximum channel activity, *i.e.* under saturating L-glutamate and glycine concentrations. This PSD-95_{c-Myc}-induced decrease in the affinity of NR1-1a/NR2A receptors for [³H]MK801 even in the presence of saturating L-glutamate and glycine, may alternatively be a due to an effect of PSD-95_{c-Myc} on the sensitivity of [³H]MK801 binding to other modulators such as protons or divalent cations.

4.4.2 The allosteric modulation of [³H]MK801 binding by L-glutamate and glycine

The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A yielded an approximate four-fold increase in the EC₅₀ for the enhancement of [³H]MK801 binding by L-glutamate compared to NR1-1a/NR2A alone. This reduced sensitivity to L-glutamate was again dependent upon the ESDV motif of the NR2A subunit since PSD-95_{c-Myc} did not decrease the sensitivity of NR1-1a/NR2A^{Trunc} receptors to L-glutamate as determined by the ratio of the percentage enhancement of [³H]MK801 binding at 1 mM : 10 μM L-glutamate (Figure 3.24). Note, NR1-1a/NR2A^{Trunc} receptors had the same EC₅₀ as NR1-1a/NR2A^{WT} when the full dose-dependent enhancement of [³H]MK801 binding by L-glutamate was carried out demonstrating that the effect of the C-terminal truncation was not due to a change in the properties of the C-terminally truncated receptor.

In addition to the decreased sensitivity of NR1-1a/NR2A receptors to L-glutamate, PSD-95_{c-Myc} induced a similar decrease in the sensitivity of [³H]MK801 binding to glycine. In the presence of 1 mM L-glutamate, PSD-95_{c-Myc} induced an approximate 6-fold increase in the EC₅₀ for the glycine enhancement of [³H]MK801 to NR1-1a/NR2A receptors (Table 3.4). The percentage enhancement of [³H]MK801 binding by glycine was also significantly greater to NR1-1a/NR2A receptors expressed in the presence of PSD-95_{c-Myc} compared to NR1-1a/NR2A receptors alone. However, in the presence of 10 μM 7-CK an equal percentage enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors was detected in the presence and in the absence of PSD-95_{c-Myc} demonstrating that this difference was due to the effect of residual glycine in the cell homogenate preparation and the greater sensitivity of NR1-1a/NR2A receptors to glycine in the absence of PSD-95_{c-Myc}.

[³H]MK801 binding to NR1-1a/NR2A receptors both in the presence and absence of PSD-95_{c-Myc} was significantly more sensitive to glycine compared to L-glutamate. For example, the EC₅₀s for the enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors by L-glutamate and glycine were 1800 ± 400 nM and 67 ± 30 nM respectively representing a 26-fold greater sensitivity to glycine. This is in contrast to the relative sensitivities of native NMDA receptors to L-glutamate and glycine reported by Ransom and Stec (1988) where [³H]MK801 binding was equally sensitive to L-glutamate and glycine.

The effect of PSD-95_{c-Myc} on the properties of cloned NMDA receptors was also analysed using the cell cytotoxicity assay. The results showed that PSD-95_{c-Myc} did not alter the cytotoxic effect of NMDA receptor expression in HEK 293 cells. The reduced

sensitivity of NR1-1a/NR2A receptors to L-glutamate and glycine was therefore, not significant enough to inhibit the activity of the receptor ion channel that has been proposed to result in cell cytotoxicity post-transfection.

The L-glutamate and glycine sensitivity of cloned NMDA receptors versus native NMDA receptors

The EC_{50} values determined for the L-glutamate enhancement of [^3H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} differ by approximately 10-fold and 5-fold, respectively from the EC_{50} value = $0.46 \pm 0.04 \mu M$ for the enhancement of [^3H]MK801 binding by L-glutamate to rat forebrain membranes (Table 3.4). The difference between native and cloned NMDA receptors may be explained by the presence of other NMDA receptor regulatory molecules such as polyamines in the brain preparation which may facilitate NMDA receptor channel activity. This would result in a leftward shift in the L-glutamate enhancement curve for [^3H]MK801 binding to native receptors compared to cloned NMDA receptors. In addition, PSD-95 is not the only post-synaptic density protein associated with native NMDA receptors. The presence of other regulatory proteins in the forebrain preparation may further enhance the activity of native NMDA receptor channels. In addition, the population of rat forebrain NMDA receptors is a mixture of NR1/NR2A and NR1/NR2B receptors. Chazot and Stephenson (1997a) reported that NR1/NR2A and NR1/NR2B receptor assemblies accounted for 37 % and 47 % of total heteromeric receptor complexes whereas NR1/NR2A/NR2B receptors accounted for only 6 %. The native forebrain receptor population assayed in this thesis was therefore likely to be heterogeneous with respect to the precise subunit combinations of receptor complexes. In addition, the membrane preparation was a crude P2 fraction and comprises both synaptic, extra-synaptic and intracellular receptors resulting in further heterogeneity in the association of PSD proteins with the either the NR1 or NR2 subunits. The Hill coefficient for the L-glutamate enhancement of [^3H]MK801 binding to forebrain membranes was however ~ 1.00 suggesting that the receptor population was homogeneous with respect to its sensitivity to L-glutamate. To determine whether the difference in sensitivity to L-glutamate between cloned NR1-1a/NR2A receptors and native rat forebrain receptors was not due to the presence of NR2B in the forebrain membrane preparation, a full characterisation of the L-glutamate sensitivity of [^3H]MK801 binding to NR1-1a/NR2B and NR1-1a/NR2A/NR2B receptors would have to be performed. Unfortunately the [^3H]MK801 binding to NR1-1a/NR2B receptors yielded low signal:noise ratios such that

the allosteric modulation of [³H]MK801 binding by L-glutamate and glycine could not be studied. This was perhaps due to the reported three- to five-fold lower peak open probability for NR1-1a/NR2B compared to NR1-1a/NR2A (Chen *et al.*, 1999).

The EC₅₀ value for native forebrain NMDA receptors is 10-fold greater than that found by Ransom and Stec (1987) for the L-glutamate enhancement of [³H]MK801 binding to rat cerebral cortical membranes, *i.e.* 0.05 μM, but it is more similar to the value found by Foster and Wong (1987), *i.e.* 0.17 μM. These differences are unlikely to be due to a higher sensitivity of cortical receptors to L-glutamate compared to the combined population of forebrain NMDA receptors since large variations in the EC₅₀s for the L-glutamate enhancement of [³H]MK801 binding to the different areas of the forebrain have not been observed (Monaghan, 1991). Instead, this discrepancy is perhaps due to differences in the conditions of the radioligand binding assay. For example, in the study by Ransom and Stec (1988) cortical membranes were prepared in 5 mM Tris-HCl, pH 7.5 and incubations were carried out for 1 h at RT. In this study, forebrain membranes were prepared in 50 mM Tris-citrate pH 7.1, 5 mM EDTA, 5 mM EGTA and incubations were carried out for 2 h at RT. The differential effects of pH, incubation times and buffer composition on [³H]MK801 or L-glutamate binding may result in these different EC₅₀s for L-glutamate.

It was not possible to determine reproducibly the enhancement of [³H]MK801 binding to rat forebrain membranes by glycine due to the difficulties in removing residual glycine from the membrane preparation. However, the glycine stimulation of [³H]MK801 binding to native and cloned receptors was compared by including the glycine site antagonist, 7-CK, to displace the binding of basal levels of glycine to the receptor. The EC₅₀ values determined in the presence of 10 μM 7-CK for the glycine enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} were approximately 10-fold and 5-fold greater than that for rat forebrain membranes respectively. This is similar to the corresponding profile of EC₅₀s determined for the enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} by L-glutamate, *i.e.* approximately 15-fold and 4-fold greater than that for rat forebrain membranes respectively. Thus, cloned NR1-1a/NR2A receptors displayed a similar decrease in sensitivity to both L-glutamate and glycine compared to native NMDA receptors. The decreased sensitivity of cloned receptors to glycine is in agreement with previous studies that have reported a lower affinity of cloned receptors for glycine compared to native NMDA receptors (Grimwood *et al.*, 1995b; Baron *et al.*, 1996; Hofner and Wanner, 1997; Chazot *et al.*, 1998; Chopra *et al.*, 2000).

Because native receptors are associated with proteins of the post-synaptic density, in particular PSD-95, one may have expected that the properties of cloned NR1-1a/NR2A receptors expressed in the presence of PSD-95 would be more representative of their native counterparts. However, NR1-1a/NR2A receptors were even less sensitive to L-glutamate and glycine in the presence of PSD-95 suggesting that the co-expression of PSD-95 in HEK 293 cells did not result in the reconstitution of receptors with properties more like native forebrain receptors. This does not mean that the effect of PSD-95 is not relevant *in vivo*. Rather, it highlights the fact the properties of cloned receptors are not necessarily representative of their native counterparts and that even in the presence of PSD-95, without the contribution of all the other proteins of the PSD, their properties are still not representative of native NMDA receptors.

The effects of PSD-95 on the L-glutamate sensitivity of [³H]MK801 binding to cloned NMDA receptors compared to L-glutamate-induced receptor currents

Once the effect of PSD-95 on the L-glutamate and glycine sensitivity of [³H]MK801 binding had been characterised, transfected HEK 293 cells were prepared for electrophysiological analyses to determine whether PSD-95 also decreased the sensitivity of NMDA receptor current responses to L-glutamate and glycine. At the time of writing, these studies are ongoing.

The effects of PSD-95 on the electrophysiological properties of cloned NR1-1a/NR2B receptors have however been studied by Yamada *et al.* (1999). They showed that the current responses of NR1-1a/NR2B receptors expressed in *Xenopus* oocytes were less sensitive to L-glutamate in the presence of PSD-95 and are thus in agreement with the findings described in this thesis. The EC₅₀ values obtained by Yamada *et al.* (1999) were 5.76 μ M and 1.39 μ M in the presence and absence of PSD-95 respectively. These values are in good agreement with the corresponding EC₅₀ values of $7.8 \pm 1.7 \mu$ M and $1.8 \pm 0.4 \mu$ M determined here. It is surprising that the EC₅₀ values obtained for the L-glutamate enhancement of [³H]MK801 radioligand binding were comparable to those reported by Yamada *et al.* (1999). Both experiments measure the effect of L-glutamate on the opening of the NMDA receptor channel but utilise very different methods of analysis. The measurement of ionic currents in response to the application of L-glutamate assays the peak current amplitude mediated by activated NMDA receptors. This is determined immediately upon the application of L-glutamate. In contrast, [³H]MK801 radioligand binding assays measure the ability of L-glutamate to open the ion channel and allow [³H]MK801 access

to its binding site within the channel pore. [³H]MK801 binding is determined by a 2 h incubation at RT. The receptors in the radioligand binding assay are therefore exposed to L-glutamate for a longer period of time compared to receptors analysed by electrophysiological methods. It is surprising that altered deactivation and desensitization kinetics of the receptor between the two experiment paradigms does not result in different EC₅₀ values.

4.4.3 Is the reduced sensitivity of NR1-1a/NR2A receptors to L-glutamate and glycine due to a PSD-95-mediated decrease in the affinity for L-glutamate and glycine?

The PSD-95-mediated decrease in the sensitivity of [³H]MK801 binding to L-glutamate or glycine may be a secondary effect resulting from a decrease in the affinity of NR1-1a/NR2A receptors for L-glutamate and glycine. The affinity of L-glutamate and glycine to NR1-1a/NR2A receptors was therefore measured by [³H]CGP39653 and [³H]MDL 105,519 displacement assays respectively.

[³H]CGP 39653 radioligand binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc} revealed that PSD-95_{c-Myc} did not significantly alter the affinity of the receptor for [³H]CGP 39653. K_D values were consistent with previously published data for [³H]CGP39653 binding to cloned NR1/NR2A receptors (6.0 nM; Kendrick *et al.*, 1996) and to native rat forebrain NMDA receptors (6.2 ± 0.6 nM; Sills *et al.*, 1991).

PSD-95 does not decrease the affinity of NR1-1a/NR2A receptors for L-glutamate

The displacement of [³H]CGP 39653 binding from NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} by L-glutamate yielded K_i values of 71 ± 18 nM and 81 ± 13 nM respectively, showing that PSD-95_{c-Myc} did not alter the affinity of the receptor for L-glutamate in this assay. Both curves were best-fit by a single binding site which is in contrast to the two sites previously reported for the L-glutamate displacement of [³H]CGP 39653 binding to NR1-1a/NR2A receptors (Laurie and Seeburg, 1994; Kendrick *et al.*, 1996). Interestingly, in these published studies, both [³H]L-glutamate and [³H]CGP 39653 binding to NR1-1a/NR2A receptors expressed in HEK 293 cells were best fit by a single site binding model, whereas the displacement of [³H]CGP39653 with L-glutamate and the displacement of [³H]L-glutamate with CGP 39653 were both best-fit by a two site model with Hill coefficients of 0.61 and 0.70 respectively (Laurie and

Seeburg, 1994; Kendrick *et al.*, 1996). Assuming there at least two NR2A subunits per functional receptor (Chazot *et al.*, 1994; Wafford *et al.*, 1993; Behe *et al.*, 1995; Premkumar and Auerbach, 1997; Laube *et al.*, 1998; Hawkins *et al.*, 1999), one would predict that there are two L-glutamate recognition sites per receptor complex. The binding of either L-glutamate or CGP 39653 to one recognition site may allosterically modulate the other recognition site such that two distinct L-glutamate or CGP 39653 binding sites are detected by competition assay. A possible explanation for the difference between the results of Kendrick *et al.* (1996) and those in this thesis with respect to whether the L-glutamate binding is best fit by a one or two site model, is that in this study 10 μ M 7-CK was included in the [^{3} H]CGP 39653 displacement assay. Even though the binding of 7-CK is thought to have minimal allosteric effects on the NMDA receptor and to simply antagonise glycine binding (Kemp and Leeson, 1993; Grimwood *et al.*, 1993), it may modulate the L-glutamate binding sites such that L-glutamate binds to both recognition sites with equal affinity. The resulting K_i values were intermediate between the high and low affinity sites detected by Kendrick *et al.* (1996), *i.e.* 5 nM and 280 nM, and were more similar to the K_D values reported for [^{3} H]L-glutamate binding to NR1-1a/NR2A receptors (102 nM, Laurie and Seeburg, 1994; 45 nM, Kendrick *et al.*, 1996).

The inclusion of 7-CK in the [^{3} H]CGP39653 binding assay was not ideal because the change in the L-glutamate sensitivity of [^{3} H]MK801 binding to NR1-1a/NR2A receptors induced by PSD-95_{c-Myc} was initially determined in the presence of 30 μ M glycine. Many groups have reported allosteric coupling between the L-glutamate and glycine binding sites (Fadda *et al.*, 1988; Monaghan *et al.*, 1988; Ransom and Stec, 1988; Compton *et al.*, 1990; Kemp and Priestley, 1991). For example, the presence of glycine enhances the sensitivity of [^{3} H]MK801 binding to L-glutamate (Ransom and Stec, 1988). The receptors present in the [^{3} H]MK801 enhancement assay and in the [^{3} H]CGP 39653 competition assay may therefore have been in a different state of allosteric modulation. Consequently, the PSD-95-induced decrease in the sensitivity of NR1-1a/NR2A receptors to L-glutamate may not have been apparent in [^{3} H]CGP 39653 competition assays due to the absence of glycine. It was important however, to completely inhibit glycine binding to the receptor. The L-glutamate recognition site is allosterically modulated by glycine such that [^{3} H]CGP 39653 binding was not detectable in the presence of 30 μ M glycine. Furthermore, as shown in Table 3.5, specific [^{3} H]CGP 39653 binding was doubled in the presence of 10 μ M 7-CK demonstrating the inhibitory effect of basal levels of glycine in the cell homogenate. It was therefore preferable to inhibit the binding of residual glycine

altogether, especially since PSD-95_{c-Myc} altered the sensitivity of NMDA receptors to glycine.

Although it is not known whether 7-CK decreases the effect of PSD-95_{c-Myc} on the sensitivity of NR1-1a/NR2A receptors to L-glutamate, these results suggest that PSD-95_{c-Myc} did not alter either the affinity of NR1-1a/NR2A receptors for either the competitive glutamate site antagonist, [³H]CGP 39653, or L-glutamate.

PSD-95 does not decrease the affinity of NR1-1a/NR2A receptors for glycine

The displacement of [³H]MDL 105519 radioligand binding by glycine determined in the presence of 1 mM L-glutamate revealed that PSD-95_{c-Myc} did not alter the affinity of NR1-1a/NR2A receptors for glycine. In both cases however, the displacement of [³H]MDL 105519 with glycine was best fit by a two site binding model. This is contrast to previously published results that describe a one site fit for the displacement of [³H]MDL 105519 from NR1-1a/NR2A receptors by glycine (Hill coefficient = 1.0, Chazot *et al.*, 1998). Furthermore, [³H]glycine binding to NR1-1a/NR2A receptors has been shown to be best fit by a one site model (Laurie and Seeburg, 1994). The apparent $K_i = 1.9 \pm 1.0 \mu\text{M}$ determined in this study for the high affinity glycine binding site is closest to the affinity constants of 3.9 μM and 3.7 μM determined for glycine by [³H]MDL 105,519 competition assay (Chazot *et al.*, 1998) and by [³H]5,7-dichlorokynurenate competition assay (Laurie and Seeburg, 1994) respectively. However, an additional low affinity site with a $K_i = 34 \mu\text{M}$ was detected in this study. A possible explanation for this two site fit is that the displacement of [³H]MDL 105519 by glycine was carried out in the presence of 1 mM L-glutamate. Although L-glutamate does not influence the binding of [³H]MDL 105,519 to either cloned NR1-1a/NR2A receptors or native NMDA receptors (Baron *et al.*, 1996; Chazot *et al.*, 1998), L-glutamate is known to allosterically modulate glycine binding (Ransom and Stec, 1988; Kessler *et al.*, 1989; Kemp and Leeson, 1993). In general, L-glutamate increases the affinity of NMDA receptors for glycine. Since there are thought to be at least two glycine binding sites per receptor complex due to the co-assembly of at least two NR1 subunits with two NR2 subunits (Behe *et al.*, 1995; Premkumar and Auerbach 1997; Laube *et al.*, 1998), it is possible that L-glutamate altered the characteristics of one glycine binding site resulting in the detection of an additional low affinity site in the presence of 1 mM L-glutamate. Alternatively, the presence of L-glutamate may alter the affinity of assembled NR1-1a/NR2A receptors, but not unassembled NR1-1a subunits, for glycine resulting in a heterogenous population of

receptors. The presence of L-glutamate may also allosterically modulate the receptor such that the two glycine recognition sites display negative co-operativity. Nevertheless PSD-95_{c-Myc} did not alter the affinity of NR1-1a/NR2A receptors for glycine at either recognition site.

The interpretation of the effect of PSD-95 on the affinity of NR1-1a/NR2A receptors for glycine is complicated by the presence of unassembled NR1 subunits which may have masked an effect of PSD-95 on the affinity of assembled receptors for glycine. Because [³H]MDL 105,519 binds with equal high affinity to assembled and unassembled NR1 subunits (Chazot *et al.*, 1998), the affinity constants for glycine will be determined for the glycine binding to both unassembled and assembled NR1 subunits. One would predict that a PSD-95-induced decrease in the affinity of NR1-1a/NR2A receptors for glycine would only be observed for NR1 subunits associated with PSD-95, *i.e.* NR1 subunits assembled with NR2A. Therefore, a significant pool of unassembled NR1 subunits, not associated with PSD-95, may have obscured a decrease in the affinity of assembled NR1-1a/NR2A receptors for glycine. Ideally, a glycine site ligand selective for heteromeric NR1-1a/NR2A receptors would be used to assay the effect of PSD-95 on the affinity of NMDA receptors for glycine. The glycine site antagonist and novel photoaffinity label, CGP 61594, displays selectivity for NR1/NR2B receptors over NR1/NR2A and NR1/NR2D receptors, but an antagonist demonstrating selectivity for assembled NR1 subunits over unassembled NR1 is not yet available (Honer *et al.*, 1998).

4.4.4. Do the apparent affinities (EC₅₀s) for L-glutamate and glycine correlate with their inhibition constants (K_is)?

A comparison of the EC₅₀ values for the L-glutamate and glycine enhancement of [³H]MK801 binding with the corresponding K_i values determined by competition assay revealed significant differences between the profile for L-glutamate and glycine (Table 4.5).

Ligand	± PSD-95	EC ₅₀ (nM)	K _i (nM)
L-glutamate	- PSD-95	1800 ± 400	81 ± 13
L-glutamate	+ PSD-95	7800 ± 1700	71 ± 18
glycine	- PSD-95	67 ± 30	site 1: 1900 ± 1000 site 2: 34000 ± 17000
glycine	+ PSD-95	265 ± 46	site 1: 1900 ± 1300 site 2: 30000 ± 19000

Table 4.5 A summary of the parameters for L-glutamate and glycine binding to NR1-1a/NR2A receptors ± PSD-95_{c-Myc}

EC₅₀ values were determined by the allosteric modulation of [³H]MK801 binding. K_i values for L-glutamate and glycine were determined by displacement of [³H]CGP 39653 and [³H]MDL 150,519 respectively.

The EC₅₀ values obtained for the allosteric modulation of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} (7800 nM and 1800 nM respectively) by L-glutamate are approx 110 times and 20 times greater than the corresponding K_i values for L-glutamate (71 nM and 81 nM respectively). A difference of this magnitude between the EC₅₀ value for agonist potency and the K_i value for agonist binding is a common feature of the agonist profiles at other ligand-gated ion channels. For example, the EC₅₀ for GABA mediated $\alpha 1\beta 3\gamma 2$ receptor currents was 8,000 nM whereas the K_i determined by inhibition of [³H]muscimol binding was 21 nM (Ebert *et al.*, 1997). Likewise, the EC₅₀ for nicotine-induced nAChR currents was 20,000 nM whereas the K_i value determined by inhibition of [³H]nicotine binding was 2 nM (Badio and Daly, 1994). A possible explanation for these differences is that the K_i values determined by radioligand binding assay measure the true affinity of the receptor for the agonist, whereas EC₅₀s determined by functional assays or by pseudo functional assays, *e.g.* the enhancement of [³H]MK801 binding by L-glutamate, reflect activation of the entire receptor/ion channel complex. If this is the case then the difference should be observed only for agonists and not antagonists. Indeed Ebert *et al.* (1997) found that the K_i values for GABA receptor antagonists had similar affinities in both binding assays and functional assays.

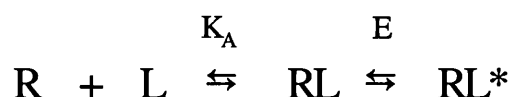
One must also consider that the assay conditions are very different between the [³H]CGP39653 and [³H]MK801 radioligand binding assays. The incubation times, incubation temperatures, buffer composition, and buffer pH are modified in the

[³H]CGP39653 binding assay to achieve optimal radioligand binding. The kinetics of L-glutamate binding to its recognition site may therefore be very different between the two assays. Furthermore, previous studies into the properties of the L-glutamate recognition site have shown that agonist and antagonist affinities measured by radioligand binding assays can be very different depending on whether they are determined by the displacement of a radiolabelled agonist or a radiolabelled antagonist (Kendrick *et al.*, 1996; Sills *et al.*, 1991; Laurie and Seeburg, 1994). For example, the K_D for [³H]CGP39653 binding to NR1-1a/NR2A receptors determined by saturation binding was 6 nM, whereas the displacement of [³H]glutamate by CGP 39653 was best fit by a two site binding model with K_I values of 40 nM and 750 nM (Kendrick *et al.*, 1996). Therefore, NR1-1a/NR2A receptors may display a increased affinity for L-glutamate as determined by [³H]CGP39653 competition assay compared to the functional affinity for L-glutamate determined by the enhancement of [³H]MK801 binding. The EC_{50} value for the enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors (1.8 μ M) was in good agreement with the values reported for L-glutamate-induced current responses mediated by NR1-1a/NR2A receptors expressed in *Xenopus* oocytes (3.7 μ M, Ishii *et al.*, 1993; 2.9 μ M, Anson *et al.*, 1998). This suggests that the affinities for L-glutamate determined by competition assay are overestimates of the actual L-glutamate affinities displayed by NR1-1a/NR2A receptors $\pm$ PSD-95_{c-Myc} when the cell homogenates are incubated in the conditions of the [³H]MK801 radioligand binding assay.

In contrast to the greater EC_{50} for L-glutamate compared to the K_I for L-glutamate, the EC_{50} s for the glycine enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} were 8 and 30 times lower than the corresponding K_I values respectively. This is inconsistent with the agonist profiles of other ligand-gated ion channels. The reason for this inconsistency may be related to the difference in the assay conditions between the glycine enhancement of [³H]MK801 binding and the [³H]MDL 105,519 competition assay. In addition, variations in the desensitization of the receptor channel between the two assays may contribute to the observed differences. For example, the receptors may be more desensitized in the [³H]MDL 105,519 assay resulting in a decrease in the affinity for glycine.

4.4.5 Modulation of NMDA receptors by PSD-95: Effects on channel gating or agonist binding?

The simplest mechanism, originally proposed by Del Castillo and Katz (1957), for the activation of a ligand-gated ion channel by an agonist is a two step equilibrium in which the receptor, R, first binds the agonist, L, to form an inactive agonist-receptor complex, RL. This complex then undergoes a conformational change, or isomerization, to the activated receptor, RL*.



K_A = Equilibrium constant for ligand binding

E = Equilibrium constant for the isomerization to the active state

The functional potency of the agonist, *i.e.* the EC_{50} for a dose-response relationship, reflects the activation of the entire receptor/ion channel complex and involves both agonist binding and isomerization to the active state. The microscopic equilibrium constants, K_A and E , relate to the individual components of the complete equilibrium. The K_A value describes the affinity of an agonist for its recognition site in isolation from any conformational changes associated with receptor isomerization. E describes the intrinsic efficacy of the receptor channel and reflects the equilibrium constant for the isomerization to the active state, *i.e.* channel gating.

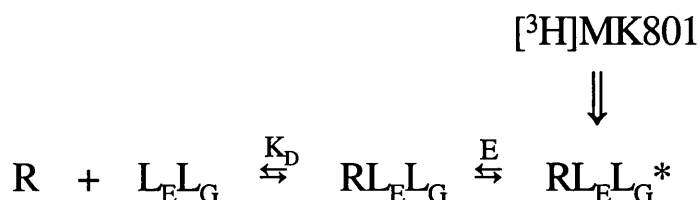
In theory an increase in the EC_{50} for the functional potency of an agonist may result from either a decrease in the affinity of the receptor for the agonist or a decrease in channel gating. In practice these two parameters are extremely difficult to separate and analyse in isolation. However, the characteristics of a particular shift in an agonist response curve give clues to whether a change in channel gating or agonist affinity has occurred. If a decrease in the channel gating of a particular receptor is induced, perhaps via an amino acid mutation, the EC_{50} for the agonist-response curve would be increased. The magnitude of this shift in EC_{50} is dependent upon the kinetic properties of the receptor. For example, if the receptor is activated by the binding of two agonist molecules, then the shift in EC_{50} is roughly proportional to the square root of the fold change in the isomerization constant, *i.e.* a 10-fold increase in the EC_{50} would require a 100-fold decrease in the isomerization

constant (Colquhoun, 1998). In addition, for dose response curves relating to a receptor activated by the binding of two agonist molecules per receptor complex, the Hill co-efficient would usually be greater than unity due to the positive co-operation of agonist binding. If a decrease in channel gating occurs it has been proposed that the Hill co-efficient for the resulting dose-response curve would then be decreased towards unity (Colquhoun, 1998; Anson *et al.*, 1998). Furthermore, a decrease in channel gating would be expected to result in a reduction in the maximum agonist-induced response unless the isomerization constant is particularly high (Colquhoun, 1998).

For the opposite scenario, *i.e.* a decrease in agonist affinity, the shift in EC_{50} would be directly proportional to the fold change in agonist affinity constants. However no change in the Hill slope or maximum response would be observed. In addition, the affinity of the receptor for an antagonist may be reduced. On the basis of these theoretical considerations the contribution of particular amino acids in some ligand gated ion channels to the formation of agonist binding sites has been investigated. For example, selected mutations in the NMDA NR2A subunit resulted in a significant increase in the EC_{50} for L-glutamate-induced NR1/NR2A receptor currents but not glycine-induced currents (Anson *et al.*, 1998). The Hill slope for the mutant receptor agonist dose-response curve was not altered, nor was there a drastic reduction in the maximum response to L-glutamate. Furthermore, these mutations resulted in a decreased affinity for the L-glutamate site antagonist, APV. The authors therefore concluded that these amino acids contributed to the L-glutamate binding site and that the increased EC_{50} was not due to a decrease in channel gating. In contrast, selected mutations in the $\alpha 1$ subunit of the human glycine receptor which also resulted in an increased EC_{50} for glycine, decreased the maximum agonist response and decreased the Hill co-efficient for the glycine dose-response curve (Lewis *et al.*, 1998). Furthermore, single channel analyses revealed an approximate 10-fold reduction in channel opening time. The authors in this case concluded that the mutation decreased channel gating rather than agonist binding.

To a certain extent these theoretical considerations can be applied to the results of this thesis. The [^3H]MK801 enhancement assays may be considered to be analogous to the stimulation of NMDA receptor currents by L-glutamate and glycine, and are thus often referred to as functional [^3H]MK801 binding assays. Indeed the EC_{50} for the L-glutamate enhancement of [^3H]MK801 binding to NR1-1a/NR2A receptors was very similar to that obtained by electrophysiological methods (see section 4.4.4). The L-glutamate and glycine enhancement of [^3H]MK801 binding would involve exactly the same isomerization steps

that result in the agonist activation of NMDA receptor currents. However, instead of measuring receptor-mediated currents by electrophysiological methods, [³H]MK801 binding is a means of detecting the concentration of active agonist-bound receptor, as shown by the following reaction scheme.



- R = Inactive, agonist-free receptor
- LE = L-glutamate
- LG = Glycine
- RL_EL_G = Agonist bound inactive receptor
- RL_EL_G* = Agonist bound active receptor
- K_D = Dissociation constant for either L-glutamate or glycine
- E = Equilibrium constant for the isomerization to the active state

A more accurate model for the L-glutamate or glycine enhancement of [³H]MK801 binding would involve multiple equilibrium constants for the association of two molecules of L-glutamate or glycine and may include additional transitions to desensitized conformational states. However, on the basis of this simple theoretical equilibrium, the observed shift in EC₅₀ for L-glutamate and glycine in the presence of PSD-95 may be due to a decrease in either channel gating or agonist affinity. With respect to the characteristics of the L-glutamate and glycine [³H]MK801 enhancement curves, it was found that in the presence of PSD-95 the Hill coefficient was reduced from 1.45 to 1.00 for L-glutamate and from 1.50 to 1.21 for glycine (in the presence of 10 μM 7-CK). Since the NMDA receptor channel is thought to be activated by a least two molecules of L-glutamate and two molecules of glycine (Patneau and Mayer, 1990; Beneveniste and Mayer, 1991), a Hill coefficient greater than unity would be expected for NR1-1a/NR2A receptors in the absence of PSD-95. Indeed, the Hill coefficients of 1.45 and 1.50 for L-glutamate and glycine respectively, are consistent with the corresponding values obtained for NR1-1a/NR2A receptors by electrophysiological methods (Anson *et al.*, 1998). The decreased Hill coefficients in the presence of PSD-95 are therefore suggestive of an effect on channel gating. The maximum response to L-glutamate, *i.e.* the maximum specific [³H]MK801

binding detected in the presence of 1 mM L-glutamate, was not significantly decreased in the presence of PSD-95, inconsistent with the predicted decrease in maximum response that would accompany a decrease in channel gating. A reduction in the maximum response resulting from a decrease in channel gating would however, only be expected if the isomerization constant for NR1-1a/NR2A receptors is particularly low.

If PSD-95 reduced the sensitivity of [^{3} H]MK801 binding to L-glutamate and glycine via a decrease in the affinity of the receptor for L-glutamate and glycine then the dissociation constants for L-glutamate and glycine binding should be increased. The fact that no change in the K_i values for either L-glutamate or glycine was observed suggests that PSD-95 does not change the agonist affinities of the receptor. It has been suggested however, that the true agonist affinity in isolation from channel gating cannot be determined precisely by radioligand binding assay due to the inevitable conformational changes that result from agonist binding (Leff *et al.*, 1990). In a radioligand binding assay the conformational changes resulting from the isomerization step pull the binding equilibrium to the right resulting in a greater agonist affinity than the true K_A . In addition, the affinity of the activated receptor for the agonist may be different from the affinity of the inactive receptor conformation. Whereas the true K_A value is a measure of the affinity of an agonist for the inactive receptor conformation, the K_i value determined in practice would also measure agonist binding to active receptor conformations and desensitized receptor conformations. Therefore, if PSD-95 had actually altered the K_i values for L-glutamate or glycine it would have been difficult to conclude that this was a direct result of a change in agonist affinity since changes in channel gating may influence K_i determinations. However, PSD-95 did not alter the K_i values for either L-glutamate or glycine suggesting that any effect on channel gating did not decrease the apparent affinity of NR1-1a/NR2A receptors for L-glutamate or glycine.

The affinities of NR1-1a/NR2A receptors for the L-glutamate and glycine site antagonists in the presence and absence of PSD-95_{c-Myc} also provide important information with respect to the effect of PSD-95 on the L-glutamate and glycine recognition sites. In theory, antagonist binding does not induce any of the conformational changes associated with channel gating. Their binding characteristics therefore provide insights into whether a change in the agonist binding site or channel gating has occurred. The fact that PSD-95 did not decrease the affinities NR1-1a/NR2A receptors for either [^{3} H]MDL 105,519 or [^{3} H]CGP39653 is further evidence to support an effect of PSD-95 on channel gating. Correlations between antagonist affinity constants and the overall properties of the agonist

recognition site must be drawn with caution however because antagonists may not bind to exactly the same amino acids as the corresponding agonist and thus a decrease in agonist affinity could in theory occur independently of a decrease in antagonist affinity. Indeed, some of the mutations in the NR1 subunit that decrease glycine potency do not affect the affinities of glycine antagonists (Kuryatov *et al.*, 1994; Wafford *et al.*, 1995; Williams *et al.*, 1996; Hirai *et al.*, 1996).

In support of an effect of PSD-95 on channel gating, the EC_{50} s for **both** L-glutamate and glycine were increased in the presence of PSD-95. Because PSD-95 interacts specifically with the NR2 subunit on the intracellular side of the membrane, it would be surprising if this interaction resulted in equivalent allosteric effects on both the L-glutamate site located on the NR2 subunit and the glycine site located on the NR1 subunit. Furthermore, it seems more probable that this PSD-95/NR2 interaction would alter NMDA receptor properties via the allosteric modulation of the channel pore region rather than extracellular domains of the receptor.

In conclusion, these results suggest that PSD-95 inhibits the channel gating of NR1-1a/NR2A receptors. This interpretation is presented with caution due to the difficulties in separating agonist binding from channel gating experimentally, especially by radioligand binding methods. The fact that the EC_{50} s for L-glutamate and glycine are so different from their corresponding K_i values suggests that the conformational state of cloned receptors is highly dependent upon the assay conditions and the type of radioligand binding assay. Furthermore, the determination of the affinity of NR1-1a/NR2A receptors for glycine was complicated by the presence of unassembled NR1-1a subunits. Future investigations into the effect of PSD-95 on the properties of cloned NMDA receptors should include single channel analysis in the presence and absence of PSD-95 which in principle could detect effects on channel gating. One would predict that alterations in channel gating should change certain kinetic parameters such as opening rate constants and mean open times.

4.4.6 Possible mechanisms for the effect of PSD-95 on the pharmacological properties of NMDA receptors

As described in section 4.4.5, the effect of PSD-95 on the pharmacological properties of cloned NR1-1a/NR2A receptors expressed in HEK 293 cells may be due to a direct conformational effect of the interaction between PSD-95 and the NR2A subunit.

Alternatively, the effect of PSD-95 on the sensitivity of L-glutamate and glycine may be mediated by another intermediate protein that associates with PSD-95. The association of PSD-95 with many signalling proteins is well established and it is thought that the recruitment of these molecules to NMDA receptors is essential for their regulation of receptor function. For example, the co-expression of an activated form of fyn tyrosine kinase and PSD-95 with NR2A has been shown to enhance the tyrosine phosphorylation of the NR2A subunit (Tezuka *et al.*, 1999). The co-expression of PSD-95 was also necessary for src-mediated potentiation of NMDA receptor-mediated currents (Liao *et al.*, 2000). Furthermore, PSD-95 interacts with the PKA-binding protein, AKAP79, the phosphatase enzyme, ζ /RPTP β , and other members of the src tyrosine kinase family (Kawachi *et al.*, 1999; Tezuka *et al.*, 1999; Colledge *et al.*, 2000). Although the precise complement of protein kinases and phosphatases that are expressed in HEK 293 cells is not known, the presence of regulatory proteins in HEK 293 cells has been demonstrated. For example, the activation of cloned mGluR5 α expressed in HEK 293 cells modulates the activity of co-expressed NR1-1a/NR2A NMDA receptors demonstrating that at least a subset of signalling proteins is present in HEK 293 cells (Collett and Collingridge, 2000). The modulation of NMDA receptor properties by PSD-95 may therefore occur via the recruitment of endogenous protein phosphatases or protein kinases such as PKC, PKA or tyrosine kinases to the receptor channel resulting in a change in the phosphorylation state of the receptor. Alternatively, PSD-95 may prevent the background phosphorylation of NMDA receptors that has been previously detected for NR1-1a subunits expressed in HEK 293 cells (Tingley *et al.*, 1997). Indeed, the co-expression of PSD-95 with ζ 1/ ϵ 2NMDA receptors in *Xenopus* oocytes abolished the potentiation of NMDA receptor currents evoked by PKC and insulin (Yamada *et al.*, 1999).

Further insights into the possible mechanism for the effect of PSD-95 on the pharmacological properties of cloned NMDA receptors were gained by co-expressing PSD-95_{c-Myc} with NMDA receptors composed of alternatively spliced NR1 subunits. The NR1 subunit is known to contain a number of consensus sites for phosphorylation, some of which have been identified in the C1 exon (Tingley *et al.*, 1997). Furthermore, the presence of either the N1 or C1 exon is known to regulate the potentiation of NMDA receptors by PKC (Grant *et al.*, 1998; Logan *et al.*, 1999). However, the PSD-95_{c-Myc}-induced decrease in the L-glutamate sensitivity of NMDA receptors was not dependent upon the C1 exon of the NR1-1a subunit (Figure 3.32). Therefore, if PSD-95_{c-Myc} alters the radioligand binding properties of NR1-1a/NR2A receptors via a change in the

phosphorylation state of the receptor, then the C1 exon of the NR1-1a subunit is not the target for this phosphorylation. In addition, the effect of PSD-95_{c-Myc} on the L-glutamate sensitivity of NR1/NR2 receptors was not dependent upon either the N1 exon or the C2 exon of the NR1-1a subunit. It is possible that other amino acids located in both the NR1 and NR2A subunits are phosphorylated or dephosphorylated in the presence of PSD-95_{c-Myc}. An attempt to investigate the phosphorylation state of the NR1-1a and NR2A subunit in the presence and absence of PSD-95_{c-Myc} by analysing the expressed NR1 and NR2A subunits using immunoprecipitation followed by immunoblotting using anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies was unsuccessful. The results were inconclusive probably due to the low affinity or specificity of the antibodies. Ideally, polyclonal antibodies raised against NMDA receptor subunit-specific peptides containing the phosphorylated amino acid would be used to analyse the phosphorylation state of NR1-1a and NR2A subunit (Tingley *et al.*, 1997). This however, requires prior knowledge of which amino acids are the targets for specific protein kinases and phosphatases. Alternatively, an effect of PSD-95 on the phosphorylation state of NR1 or NR2 subunits could be investigated by measuring the incorporation of [³²P]-orthophosphate into cloned NR1 and NR2 subunits expressed in the presence and absence of PSD-95_{c-Myc}.

4.4.7 Insights into the regulation of NMDA receptors by PSD-95 *in vivo*

The PSD-95_{c-Myc}-mediated decrease in the sensitivity of NR1-1a/NR2A receptors to L-glutamate and glycine may have important consequences on NMDA receptor function *in vivo*. However, the relevance of these effects to the properties of native NMDA receptors is dependent upon a number of factors. Firstly, it is important whether the sensitivity of native NMDA receptors to L-glutamate and glycine is actually influenced by the association of PSD-95 with the NR2 subunit. A number of studies on genetically altered mice have investigated whether the properties of native NMDA receptors are altered when the interaction with PSD-95 is disrupted. In mutant mice lacking PSD-95, the NMDA receptor channels were indistinguishable from those in wild-type mice with respect to peak currents and current/voltage relationships (Migaud *et al.*, 1998). A full characterisation of the sensitivity of these NMDA receptors to agonists, antagonists and allosteric modulators was not carried out however. Interestingly, synaptic transmission in the hippocampus of the PSD-95-knockout mice was significantly more sensitive to LTP-inducing stimuli. Furthermore, frequencies of pre-synaptic stimuli that induce LTD in wild-type mice,

actually induced LTP in the PSD-95-knockout mice. It is possible that the NMDA receptors in these mice are more sensitive to L-glutamate due to the absence of PSD-95 and that this contributes to their greater sensitivity to LTP-inducing stimuli.

The role of the association of the NR2 subunit C-termini with proteins of the PSD *in vivo* has also been investigated by the generation of mutant mice expressing C-terminally truncated NR2 subunits (Sprengel *et al.*, 1998; Mori *et al.*, 1998; Steigerwald *et al.*, 2000). In all cases, these mice exhibited deficient hippocampal synaptic plasticity consistent with a function of PSD proteins as transduction molecules in the signalling pathways connecting NMDA receptor activity to changes in synaptic efficacy. However, in addition to these changes in synaptic plasticity, Steigerwald *et al.* (2000) reported a change in the kinetics of the eEPSCs and miniature EPSCs (mEPSCs) mediated by NR1/NR2A receptors in genetically altered NR2A^{ΔC} mice such that the eEPSCs rose and deactivated more slowly in NR2A^{ΔC} mice compared to wild-type mice. Two hypotheses were provided by the authors to explain these results. Firstly, they acknowledge that the slower deactivation of mutant NMDA receptor EPSCs could be due to both an increase in the affinity of NR1/NR2A^{ΔC} receptors for L-glutamate and an increase in the efficiency of channel gating compared to wild-type. However, they discount this hypothesis on the assumption that wild-type NR1/NR2A receptors as opposed to mutant NR1/NR2A^{ΔC} would display a higher affinity for agonists resulting in faster, not slower, mutant channel kinetics. This prediction was based on results obtained for cloned glycine receptors which displayed higher agonist affinities when clustered into dense receptor rich domains (Taleb and Betz, 1994). When applied to NMDA receptors, the observations of Taleb and Betz (1994) suggest that wild-type NMDA receptors clustered by their C-termini-associated proteins would be expected to have a higher affinity for L-glutamate compared to diffusely distributed NR1/NR2A^{ΔC} receptors. Since this predicted change in L-glutamate affinity could not explain the observed change in kinetics, the authors provided an alternative explanation. They suggested that the slower kinetics of the mutant receptor EPSCs could be explained by a re-distribution of C-terminally truncated receptors to sites just outside the boundaries of the PSD due to the lack of an association with PSD proteins. These receptors may still be classified as synaptic receptors and would be activated during pre-synaptic activity. However, the greater distance of these receptors from the site of glutamate release may account for the slower kinetics of activation and deactivation. Although the results in this thesis do not discount this explanation, they suggest that the prediction by Steigerwald *et al.* (2000) that wild-type receptors would be more likely to display a higher affinity for

L-glutamate compared to truncated NR1/NR2A^{ΔC} receptors may not be correct. Indeed, in contrast to the increased agonist affinity of glycine receptors expressed either in the presence of gephyrin or at high receptor densities (Takagi *et al.*, 1992; Taleb and Betz, 1994), the effect of PSD-95 on cloned NR1-1a/NR2A receptors was to decrease the agonist sensitivity. Therefore, wild-type NMDA receptors in association with PSD-95, may display a decreased sensitivity to L-glutamate compared to NR1/NR2A^{ΔC} receptors in the mutant mice. Consequently, an increase in either the affinity of NR1/NR2A^{ΔC} receptors for L-glutamate or an enhancement of channel gating could indeed explain the slowed kinetics displayed by mutant NR1/NR2A^{ΔC} receptors. It would be interesting to compare the L-glutamate and glycine sensitivity of native NR1/NR2A receptors that are bound to PSD-95 with the sensitivity of the NR1/NR2A^{ΔC} receptors from NR2A^{ΔC} mice that do not associate with PSD-95. The *in vivo* observations of Steigerwald *et al.* (2000) may prove to be directly related to the *in vitro* effects of PSD-95 on the L-glutamate and glycine sensitivity of cloned NMDA receptors.

The potential effect of PSD-95 on the properties of native NMDA receptors may only be important *in vivo* if the association of PSD-95 with the NR2 subunit is dynamic or if the association of native NMDA receptors with PSD-95 is regionally heterogeneous. It is possible that regulation of the association between PSD-95 and NMDA receptors *in vivo* is a means by which the properties of NMDA receptors in the post-synaptic membranes of excitatory neurons can be modulated. Alternatively, if all NMDA receptors are associated with PSD-95 and if this association is not regulated, then the change in sensitivity to L-glutamate and glycine may only be relevant to cloned NMDA receptors expressed in the presence and absence of PSD-95. However, biochemical and ultrastructural studies suggest that native NMDA receptors are indeed heterogeneous with respect to their association with PSD-95 indicating that the effect of PSD-95 on the properties of NMDA receptors may be very relevant *in vivo*. For example, extra-synaptic receptors are presumably dissociated from the proteins of the PSD and may therefore display significantly different pharmacological properties from synaptically localised receptors. As described in section 4.2.1, the structure of the PSD is also thought to be highly dynamic suggesting that the association of PSD-95 with NMDA receptors localised within the PSD may be a regulated one. Furthermore, the biochemical studies of Sans *et al.* (2000) demonstrate that the NR2A subunit displays a preferential association with PSD-95 and PSD-93 whereas NR2B is preferentially associated with SAP102. It would therefore be interesting to determine whether the co-expression of PSD-93, SAP102 or SAP97 with cloned NR1-1a/NR2A

receptors has the same effect as PSD-95 on receptor function. It is an intriguing possibility that the differential association of NMDA receptor subtypes with the proteins of the PSD may contribute to the functional heterogeneity of NMDA receptors. The heterogeneous distribution of the PSD-95 family of proteins adds even greater complexity to the potential diversity of NMDA receptor function. For example, PSD-93 is localised exclusively to Purkinje cells of the cerebellum whereas PSD-95, SAP102, and SAP97 are all co-localised in the granule cell layer (Kim *et al.*, 1996; Brenman *et al.*, 1998, Bassand *et al.*, 1999).

A third prerequisite for the relevance *in vivo* of the PSD-95-mediated regulation of NMDA receptor function is that the concentrations of L-glutamate and glycine at the synapse fluctuate within the range of the EC₅₀s of native NMDA receptors for L-glutamate and glycine. It may be that during pre-synaptic activity, the concentration of L-glutamate and glycine would be saturating for NMDA receptors whether they are associated with PSD-95 or not. Indeed, it is debatable whether the free concentration of glycine in the extracellular space of the synapse is below saturating concentrations even when the pre-synaptic nerve is silent (reviewed in Danysz and Parsons, 1998). However, the effect of PSD-95 may be important for the length of time that receptors remain active when the concentrations of L-glutamate and glycine decline due to the activity of L-glutamate and glycine transporters. Receptors associated with PSD-95 may deactivate more quickly due to their decreased sensitivity to L-glutamate. Indeed, the kinetics of the eEPSCs mediated by native receptors associated with the proteins of the PSD did display faster kinetics compared to the truncated NR1/NR2A receptors expressed in genetically altered mice (Steigerwald *et al.*, 2000).

4.5 DEMONSTRATION OF AN INTERACTION BETWEEN NR2A SUBUNITS AND PSD-95_{c-Myc} IN TRANSFECTED HEK 293 CELLS

Since the identification of PSD-95 as an NMDA receptor-associated protein, a number of groups have demonstrated the co-association and co-localization of NMDA receptors and PSD-95 in either transfected cell lines or in yeast two-hybrid assays (Kornau *et al.*, 1995; Kim *et al.*, 1996; Bassand *et al.*, 1999). For example, the co-expression of PSD-95 with NR1-1a/NR2A receptors in COS cells results in the co-localization of all three proteins to receptor-rich domains (Kim *et al.*, 1996). However, it was important in this thesis to verify that PSD-95 does indeed co-associate with the NR2A subunit in transfected HEK 293 cells. It was also necessary to quantify the association of PSD-95 with

both NR2A^{WT} and NR2A^{Trunc} subunits since some of the effects of PSD-95 were not completely abolished by the truncation of the C-terminal ESDV motif. Co-immunoprecipitation studies revealed that indeed the NR2A^{Trunc} subunit does still interact with PSD-95 but with a decreased affinity. A precise quantification of the NMDA receptor/PSD-95 association was difficult to determine however due to the effect of PSD-95 on the detergent solubility of the NR2A subunit.

Decreased detergent solubility of cloned NMDA receptors expressed in the presence of PSD-95_{c-Myc}

Initial experiments showed that NR1-1a/NR2A and PSD-95_{c-Myc} were both successfully detergent solubilised in 1% Triton X100. However, in the presence of PSD-95_{c-Myc}, the detergent solubility of the NR2A subunit was reduced approximately 2-fold while the solubility of the NR1 subunit was unchanged. The fact that a large proportion of NR1-1a subunits are expressed as unassembled subunits and are not PSD-95_{c-Myc}-associated explains why a decrease in the detergent solubility of the NR1-1a subunit in the presence of PSD-95_{c-Myc} was not observed.

The increased resistance of NR2A subunits to detergent extraction in the presence of PSD-95_{c-Myc} may be due to the association of PSD-95 with the plasma membrane. PSD-95 is a cytosolic protein but it is modified post-translationally by the addition of palmitate to two N-terminal cysteine residues (Topinka and Brecht, 1998; Craven *et al.*, 1999). It has been proposed that via this lipid modification, PSD-95 is tethered to the plasma membrane and is thus retained in the cell membrane fraction of transfected cell homogenates (Topinka and Brecht, 1998). However, since the NR2A subunit is an integral membrane protein it is unlikely that the association of PSD-95 with the plasma membrane is responsible for the decrease in the NR2A detergent solubility. Instead, the clustering of NMDA receptors into a large macromolecular complex by PSD-95 may result in a resistance of the clustered receptors to detergent extraction. Alternatively, PSD-95 may associate with other intracellular cytoskeletal proteins which may further increase the resistance of NMDA receptors to detergent extraction. It is relevant that native NMDA receptors are more resistant to detergent extraction compared to recombinant receptors expressed in transfected HEK 293 cells (Chazot and Stephenson, 1997b). With respect to their detergent solubility therefore, NR2A subunits expressed in HEK 293 cells in the presence of PSD-95_{c-Myc} are more representative of native NMDA receptors than NR2A subunits expressed alone.

Initial immunoprecipitation studies revealed that the amount of NR1 and NR2A subunit immunoprecipitated with either anti-NR1 (911-920) or NR2A (1,381-1,394) antibodies was influenced by the PSD-95-mediated decrease in detergent solubility of NMDA receptors (Figure 3.22.2). Anti-NR2A (1,381-1,394) antibodies immunoprecipitated less NR2A subunit from HEK 293 cells transfected with NR1-1a/NR2A subunits in the presence of PSD-95_{c-Myc} compared to control transfections in the absence of PSD-95_{c-Myc} (Figure 3.30). The most likely explanation for this is the decreased detergent solubility of the NR2A subunit in the presence of PSD-95_{c-Myc}, and thus a decrease in the amount of NR2A subunit available for immunoprecipitation. In contrast, the level of NR1-1a immunoprecipitated with anti-NR1 antibodies in the presence of PSD-95_{c-Myc} was not altered, presumably because the detergent solubility of the NR1-1a subunit was not decreased by PSD-95_{c-Myc}. However, a PSD-95_{c-Myc}-mediated decrease in the amount of NR1 and NR2A subunit co-immunoprecipitated with anti-NR2A (1,381-1,394) antibodies and anti-NR1 (911-920) antibodies respectively, was observed. This suggested that PSD-95_{c-Myc} decreased the detergent solubility of both NR1-1a and NR2A when they are co-assembled into the same NR1-1a/NR2A/PSD-95 complex. It follows therefore that for the level of NR1-1a subunit detergent solubilized in the presence and absence of PSD-95 to be equal, unassembled NR1-1a as opposed to assembled NR1-1a must be preferentially solubilized in the presence of PSD-95_{c-Myc}.

Co-immunoprecipitation of NR2A^{Trunc}/PSD-95_{c-Myc} complexes

Initial experiments found that anti-NR2A (1,381-1,394) antibodies were optimal for the co-immunoprecipitation of NR2A/PSD-95_{c-Myc} complexes from transfected HEK 293 cells. The other antibodies tested either failed to co-immunoprecipitate PSD-95_{c-Myc} or demonstrated cross-reactivity with PSD-95_{c-Myc}. Unexpectedly, anti-NR1 (17-35) antibodies immunoprecipitated PSD-95_{c-Myc} from HEK 293 cells transfected with PSD-95_{c-Myc} alone (Figure 3.30). The reason for this cross-reactivity of the anti-NR1 (17-35) antibodies with PSD-95_{c-Myc} is not known and is very surprising since the antibodies are affinity purified using the NR1 (17-35) specific peptide. The anti-NR1 (911-920) antibodies did not cross react with PSD-95_{c-Myc} but they were unable to show co-immunoprecipitation of PSD-95_{c-Myc} with NR2A. This may be due to the preferential solubilization of unassembled NR1 subunits resulting in the presence of predominantly unassembled NR1 in the soluble fraction.

The immunoprecipitation of NR2A subunits from HEK 293 cells transfected with

NR1-1a/NR2A^{Trunc}, resulted in the co-immunoprecipitation of PSD-95_{c-Myc}. However, the amount of PSD-95_{c-Myc} co-immunoprecipitated with NR2A^{Trunc} was decreased compared to that co-immunoprecipitated with NR2A^{WT} suggesting that PSD-95_{c-Myc} did still associate with NR2A^{Trunc} but with a decreased affinity. This association of PSD-95_{c-Myc} with NR2A^{Trunc} may explain why the co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A receptors induced a 50 % increase in the number of [³H]MK801 binding sites. The decreased affinity of the interaction between PSD-95_{c-Myc} and NR2A^{Trunc} was still sufficient to mediate this PSD-95_{c-Myc}-induced increase in the number of [³H]MK801 binding sites to NR1-1a/NR2A^{Trunc} but was not sufficient to mediate a PSD-95_{c-Myc}-induced reduction in the sensitivity of NR1-1a/NR2A^{Trunc} receptors to L-glutamate (Figure 3.24). One would predict that if PSD-95_{c-Myc} could associate with NR1-1a/NR2A^{Trunc} enough to stabilize the receptor in the plasma membrane, it should also decrease the sensitivity of the receptor to L-glutamate at least to an intermediate level. It may be that the ESDV motif of the NR2A subunit is necessary to mediate the PSD-95_{c-Myc}-induced decrease in L-glutamate sensitivity even if PSD-95_{c-Myc} is associated with the receptor. The co-expression of PSD-95_{c-Myc} with NR1-1a/NR2A^{Trunc} also prevented the appearance of the lower molecular weight NR2A immunoreactive band with M_r = 175 kDa (Figure 3.22). The C-terminal truncation of NR2A did not therefore abolish the effect of PSD-95 on the appearance of this lower molecular weight band. Whatever the mechanism responsible for the disappearance of the lower molecular weight band, it is clear that either the association of PSD-95_{c-Myc} with NR2A^{Trunc} is sufficient to mediate this effect or it is not dependent upon an interaction between PSD-95_{c-Myc} and NR2A.

The interaction of NR2A^{Trunc} with PSD-95_{c-Myc} disagrees with the findings of yeast two-hybrid protein-protein interaction assays and the *in vitro* fusion protein pull down assays where a requirement of the tSXV motif for the interaction between C-terminal peptides and the PDZ domains of PSD-95 was shown. For example, Kornau *et al.* (1995) reported that an NR2B truncated C-terminal polypeptide lacking its ESDV amino acids did not interact with full length PSD-95 when both constructs were co-expressed as fusion proteins in a yeast two-hybrid gene reporter assay. In a similar study, Kim *et al.* (1995) demonstrated that substitution of the valine residue in the ETDV motif of the Kv1.4 K⁺ channel with alanine or glutamine completely abolished the interaction between Kv1.4 and PSD-95. Substitution of the threonine residue with alanine also completely abolished the interaction although a serine could be tolerated at this position. Additional studies have identified that the tSXV motifs of other PSD-95 and *dlg* ligands such as neuroligin1,

HTLV-1 Tax and adenovirus E4 ORF1 proteins are required to mediate an interaction (Bassand *et al.*, 1999). However, the association of NR2A^{Trunc} with PSD-95_{c-Myc} is not totally surprising since the participation of other amino acid sequences in the association of PSD-95 with NR2 subunits has been reported previously. For example, the co-expression of a 600 amino acid C-terminal fragment of NR2B lacking its C-terminal seven amino acids with PSD-95 as fusion proteins in the yeast two hybrid system resulted in reporter gene activation whereas the co-expression of a 100 amino acid C-terminal fragment lacking these seven amino acids did not (Kornau *et al.*, 1995). In addition, a chimeric NR1-4b subunit containing the NR2A C-terminal five amino acids did not interact with PSD-95 in yeast suggesting that other upstream sequences present in the NR2A subunit were also necessary to mediate the interaction (Bassand *et al.*, 1999).

The difference between NR2A and other PSD-95-associated proteins with respect to the amino acid sequences necessary to mediate an interaction with PSD-95 may be due to differences in protein-protein interaction assays. The folded structures of the C-terminal polypeptides and PSD-95 PDZ domains used to assay the specificity of the interaction between many of the PSD-95-associated proteins and PSD-95 are likely to be different from the structure of the native protein because in yeast two-hybrid assays they are expressed as fusion proteins. The three dimensional structure of the full length NR2A subunit and PSD-95 expressed in HEK 293 cells is likely to be more representative of the native proteins and may therefore explain the association of NR2A with PSD-95 independently of the ESDV C-terminal amino acids. Alternatively, the difference between this study and those just described maybe that additional sequences present in the NR2A subunit contribute to the interaction with PSD-95 whereas for the other PSD-95 ligands, the tSXV motif may be the only sequence present that constitutes a PDZ binding domain and is thus mandatory for the interaction.

The K⁺ channel subtypes, Kir 2.1 and Kir 2.3, do not have consensus tSXV motifs yet they are known to associate with PSD-95 (Cohen *et al.*, 1996; Nehring *et al.*, 2000). This highlights the complexity of C-terminal peptide-PDZ interactions. Indeed, PDZ domains have been shown to bind a number of diverse amino acid sequences such as other PDZ domains and SH3 domains and it is clear that a consensus tSXV motif is not necessarily mandatory for a protein/PDZ interaction (Songyang *et al.*, 1997). Conversely, although protein database searching has revealed an extensive list of polypeptides that terminate with the sequence tSXV it cannot be presumed that each will bind a PDZ domain (reviewed in Nagono *et al.*, 1998).

The results presented in this thesis substantiate the view that the affinity and specificity of the interaction of a particular protein with a PDZ domain is determined by both the three dimensional structure of the native protein and by the sequences upstream of a consensus tSXV motif. At least for the association of the NR2A subunit with PSD-95, a tSXV motif is not mandatory but it is required to mediate a high affinity interaction, indicating that the association between NR2A and PSD-95 is more complex than a simple tSXV/PDZ domain interaction. Future studies to elucidate the three dimensional structure of PSD-95 may endeavour to resolve the X-ray crystallographic structure of a PDZ domain in association with extended portions of the NR2A C-terminus. To date, the three-dimensional structure of the PDZ2 and PDZ3 domains of PSD-95 have identified residues involved in the recognition of the consensus tSXV peptide motifs (Doyle *et al.*, 1996; Carbal *et al.*, 1996; Tochio *et al.*, 2000). However, further structural analyses may identify novel PDZ binding motifs formed by upstream sequences in the NR2A C-terminus and in the PSD-95 binding domains of other proteins.

4.6 THE IMPORTANCE OF RECEPTOR CLUSTERING FOR THE EFFECTS OF PSD-95_{c-Myc} ON THE BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF NMDA RECEPTORS

The clustering of NMDA receptors by PSD-95 has been shown to be dependent upon the two N-terminal cysteine residues located at amino acid positions 3 and 5. Immunocytochemical analyses of transfected COS cells revealed that mutagenesis of these cysteine residues abolished the PSD-95-induced clustering of NMDA receptors (Hsueh *et al.*, 1997). Furthermore, Hsueh *et al.* (1997) demonstrated that PSD-95 and PSD-93 could be co-immunoprecipitated from transfected COS cells, and that this co-association was dependent upon the two N-terminal cysteines. The results presented in this thesis show that indeed the effects of PSD-95 on the properties of cloned NMDA receptors were reduced by the inhibition of the clustering function of PSD-95 providing further insights into the mechanism of the modulation of NMDA receptor expression and function.

Mutation of the two N-terminal cysteine residues of PSD-95 inhibits the regulation of NMDA receptor expression and function by PSD-95

In general, the effects of PSD-95_{c-Myc} on both the expression of NR1-1a/NR2A receptors and on their radioligand binding properties were reduced but not completely

abolished by the two N-terminal cysteine mutations. The co-expression of PSD-95_{c-Myc}(C3S/C5S) with NR1-1a/NR2A receptors increased the intensity of NR2A subunit immunoreactivities but with a decreased magnitude compared to wild-type PSD-95_{c-Myc} (see results Figure 3.27). In addition, PSD-95_{c-Myc}(C3S/C5S) increased the number of [³H]MK801 binding sites detected in cells transfected with NR1-1a/NR2A receptors and decreased the sensitivity of the receptors to L-glutamate, but the percentage change was reduced compared to wild-type PSD-95_{c-Myc} (Figure 3.28 and 3.29). This suggests that the clustering function of PSD-95_{c-Myc} is required for the PSD-95_{c-Myc}-mediated modulation of cloned NMDA receptors. The reduced effects of PSD-95_{c-Myc}(C3S/C5S) compared to the wild-type PSD-95_{c-Myc} were not due to a reduced association of PSD-95_{c-Myc}(C3S/C5S) with the NR2A subunit because co-immunoprecipitation studies demonstrated that the amounts of PSD-95_{c-Myc}(C3S/C5S) and wild-type PSD-95_{c-Myc} co-immunoprecipitated with NR2A were comparable.

The reduced decrease in the L-glutamate sensitivity of NR1-1a/NR2A receptors by PSD-95_{c-Myc}(C3S/C5S) is in agreement with Yamada *et al.* (1999). They reported a reduced effect of PSD-95(C3S/C5S) compared to wild-type PSD-95 on the sensitivity of NR1-1a/NR2B currents to L-glutamate. Since the mechanism by which PSD-95 reduces the sensitivity of NMDA receptors to L-glutamate is unknown it is difficult to speculate why a clustering-deficient PSD-95_{c-Myc} does not reduce the sensitivity by as much as the wild-type PSD-95_{c-Myc}. However, the dense packing of NMDA receptors into cell surface clusters by PSD-95 may result in an inhibition of the conformational changes associated with ion channel activation. An inhibition of channel function via receptor clustering is in contrast to the increased agonist affinity of glycine receptors expressed either at high receptor densities or in the presence of the clustering protein, gephyrin (Takagi *et al.*, 1992; Taleb and Betz, 1994). It is intriguing that in addition to the dependence of functional NMDA receptor activity on secondary, tertiary and quaternary structures, the interactions between separate functional NMDA receptor complexes mediated by associated proteins, may regulate NMDA receptor function.

With respect to the enhanced expression of NR1-1a/NR2A receptors by PSD-95_{c-Myc}, the clustering of receptors by PSD-95_{c-Myc} appears to be important but not essential for this effect. If PSD-95_{c-Myc} enhances the level of NR1-1a/NR2A receptors in the plasma membrane either by preventing the degradation of receptors or by inhibiting the internalization of receptors, both the association of PSD-95_{c-Myc} with the receptor and the clustering effect of PSD-95_{c-Myc} may contribute equally to the efficiency with which

PSD-95_{c-Myc} performs this function. It can be envisaged that if PSD-95 masks an internalization sequence located in the NR1 subunit via its interaction with the NR2 subunit, the efficiency with which this sequence is obscured may be greater if many NR1-1a/NR2A receptors are clustered by an extensive lattice of multimerized PSD-95 proteins compared to single receptor complexes associated with two or three PSD-95 molecules.

The 3, 5, N-terminal cysteine residues determine the membrane distribution of PSD-95

The differential distribution of wild-type PSD-95_{c-Myc} and the PSD-95_{c-Myc}(C3S/C5S) mutant to the membrane and soluble fractions of transfected HEK 293 cells (Figure 3.25) is consistent with a study by Topinka and Bredt (1998) who demonstrated a similar difference in the distribution of wild-type PSD-95 and PSD-95(C3S/C5S) in COS cells. In addition, they found that after the extraction of cell homogenates in sodium bicarbonate buffer, pH 11, the PSD-95(C3S/C5S) mutant was totally removed from the membrane fraction whereas the distribution of the wild-type PSD-95 remained unchanged. Topinka and Bredt (1998) also suggested that palmitoylation of the PSD-95 N-terminal cysteine residues was required for the association with the plasma membrane and that the PSD-95(C3S/C5S) mutant could not associate with the membrane due to deficient palmitoylation. In contrast, Hsueh and Sheng (1999) reported that the PSD-95(C3S/C5S) mutant was associated with the membrane fraction of transfected COS cells to the same extent as wild-type PSD-95 even after extraction with sodium bicarbonate buffer, pH 11. They also argue that palmitoylation may be important for the tight association of PSD-95 with the cell membrane but it is not mandatory. The results presented in this thesis show that after homogenisation of transfected HEK 293 cells, the PSD-95_{c-Myc}(C3S/C5S) mutant is still associated with the membrane fraction and agrees with Hsueh and Sheng (1999) in this respect. However, in contrast to the study by Hsueh and Sheng (1999), a preferential but not total redistribution of PSD-95_{c-Myc}(C3S/C5S) to the cytoplasm was observed suggesting that the cysteine residues are important for a tight association of PSD-95 with the plasma membrane. Whether this association with the membrane is specifically due to palmitoylation of both cysteine residues cannot be deduced from these results.

The association of PSD-95_{c-Myc}(C3S/C5S) with NMDA receptors is in agreement with Hsueh and Sheng (1999), but not with Topinka and Bredt (1998). Hsueh and Sheng

(1999) showed that PSD-95(C3S/C5S) could associate with Kv1.4 K⁺ channels but could not form a ternary complex with a third PDZ interacting protein, fascilin1. The inability of PSD-95(C3S/C5S) to form a ternary complex explains why PSD-95(C3S/C5S) is able to bind Kv1.4 but is unable to cluster the channel into receptor rich domains. In contrast, Topinka and Brecht (1998) suggest that PSD-95(C3S/C5S) cannot associate with the K⁺ channel and conclude that palmitoylation of the two N-terminal cysteine residues of PSD-95 is required for the association of PSD-95 with the Kv1.4 channel and for the clustering in heterologous cells.

The mechanism of receptor-clustering by PSD-95

The precise mechanism for the clustering of NMDA receptors by PSD-95 has not been fully resolved. Sheng and colleagues originally suggested that PSD-95 molecules could form homo-multimers via the formation of disulphide bonds between the N-terminal cysteine residues (Kim *et al.*, 1996). Their analysis of COS cells transfected with PSD-95 by SDS-PAGE under non-reducing conditions revealed that a significant proportion of PSD-95 immunoreactivity appeared as bands with molecular weights of multiples of 90 kDa, whereas the higher molecular weight bands were not detected for the PSD-95_{c-Myc}(C3S/C5S) mutant. However in reply, Topinka and Brecht (1998) reported that these PSD-95 multimers found under non-reducing conditions were artefacts due to the formation of disulphide bonds *in vitro*, possibly by the freezing of transfected cell membranes. When cell homogenates were analysed immediately, the PSD-95 multimers were not detected (Topinka and Brecht, 1998). In Figure 3.26, the majority of PSD-95_{c-Myc} was present as a 100 kDa monomer when transfected HEK 293 cell homogenates were analysed under non-reducing conditions although some minor higher molecular weight immunoreactive species were seen. This is in agreement with Topinka and Brecht (1998), despite the fact that the HEK 293 cell membranes analysed in Figure 3.26 were frozen prior to SDS-PAGE analysis. The results here therefore support the view that the mechanism for the clustering of NMDA receptors by PSD-95 is not primarily via the formation of disulphide bonds between the N-terminal cysteine residues of PSD-95. Rather than mediating an interaction between separate PSD-95 proteins, the cysteine residues may be involved in the formation of intramolecular tertiary structures required to mediate homo-multimerization. This may occur either via the formation of disulphide bonds or via post-translational modifications. The characterisation of other receptor-associated proteins have identified a variety of motifs required for the clustering of their respective receptor

proteins expressed in heterologous cells. For example, both rapsyn and PICK1 display coil-coil domains that are required for nAChR and AMPA receptor clustering, respectively (Ramarao and Cohen, 1998; Xia *et al.*, 1999). In contrast, the 55 kDa AMPA receptor-clustering protein, Narp, clearly migrates with $M_r > 200$ when assayed by SDS-PAGE in non-reducing conditions, consistent with the formation of cysteine linked multimers (O'Brien *et al.*, 1999). The mGluR5- and mGluR α -clustering protein, Homer 1c, has a COOH-terminal leucine zipper motif that is required for self-multimerization (Tadokoro *et al.*, 1999). The relative contribution of disulphide bonds, N-terminal palmitoylation, and as yet unidentified secondary structures of PSD-95 to the clustering of NMDA receptors remains to be resolved.

4.7 SUMMARY AND FUTURE PERSPECTIVES

In summary, the main results of this thesis demonstrate that PSD-95 does effect the biochemical and pharmacological properties of cloned NMDA receptors. These results imply that the association of NMDA receptors with proteins of the PSD, in particular PSD-95, may be an important determinant for both the expression and function of native NMDA receptors at synaptic sites. Although the relevance of these findings to the function of PSD-95 *in vivo* remains to be ascertained, the effects of PSD-95 on the properties of receptors expressed in mammalian cells appear to be consistent, in terms of both receptor expression and receptor function, with *in vivo* studies investigating the properties of C-terminal truncated NR1/NR2A receptors which are disconnected from the proteins of the PSD.

This study also demonstrates that the properties of cloned NMDA receptors may be significantly different from their native counterparts. The co-expression of PSD-95, a protein thought to be associated with a large proportion of NMDA receptors *in vivo*, with NR1/NR2A receptors in HEK 293 cells altered the expression of these receptors, their radioligand binding properties, and their detergent solubility. The association of other interacting proteins may also have profound effects on the properties of the receptor ion channel. Caution should therefore be exercised in the correlation of the properties of neurotransmitter receptors expressed in isolation in non-neuronal cells to the properties of native receptors localised at synapses.

Now that the majority of the proteins associated directly and indirectly with the NMDA receptor have been identified (Husi *et al.*, 2000), future investigations are likely to

concentrate on the role of these proteins in the native system. In particular, the dissection of the signalling pathways responsible for the NMDA receptor-mediated changes in synaptic efficacy may have the greatest impact on our understanding of the function of NMDA receptor-associated proteins. It is important however, to consider the direct consequences of the interaction between NMDA receptors and the proteins of the PSD on the expression and function of native NMDA receptors. Approaches to elucidate the functional significance of an interaction between NMDA receptors and PSD-95 should involve a combination of both *in vitro* and *in vivo* studies. The aim of this thesis was to focus solely on the interaction between PSD-95 and the NMDA receptor in isolation from the many other NMDA receptor-associated proteins and PSD-95-interacting proteins. Of course, the effects of PSD-95 on the properties of NMDA receptors must now be characterised *in vivo*. However, it is important to consider their effect on the properties of NMDA receptors in isolation to avoid the problems of functional redundancy. Therefore, the expression of cloned NMDA receptors in non-neuronal cell lines may be a suitable system to investigate further the mechanisms for the effect of PSD-95 on both the expression and function of NMDA receptors. For example, it would be interesting to determine, perhaps via the quantitative analysis of the incorporation of [³²P]-orthophosphate, whether the co-expression of PSD-95 in HEK 293 cells alters the phosphorylation state of either the NR1 or the NR2A subunit. Indeed, studies of this kind may identify novel mechanisms for the regulation of NMDA receptor properties. It would also be interesting to analyse the effect of the co-expression of cloned NMDA receptors with the other members of the PSD-95 family. Differential effects would suggest that regional or preferential interactions of NMDA receptors with PSD-95 family proteins contribute to the functional heterogeneity of native NMDA receptors. In addition, a detailed analysis of the regulation of NMDA receptor expression in HEK 293 cells may also identify the mechanism responsible for the enhanced expression of cloned receptors in the presence of PSD-95. It is possible that PSD-95 regulates the internalization of cell surface receptors, the proteolysis of receptor subunits, the cell surface targeting of assembled receptors, or the retention of either NR1 or NR2 inside the cell.

These *in vitro* studies would form the basis for a more detailed investigation performed in neuronal cultures or brain slices. *In vivo* studies should aim to determine whether a disrupted interaction with PSD-95 alters either the expression profile of NMDA receptors or the functional properties of receptors, in particular their sensitivity to L-glutamate and glycine. The association of NMDA receptors with PSD-95 and other PSD

proteins could be inhibited either by the infusion or the viral transfection of neurons with peptides corresponding to the interacting domains of the NR2 subunit C-terminus or the PDZ domains of PSD-95. Alternatively, the infusion of antibodies raised against these interacting domains could be used to inhibit the interaction *in vivo*. The cell surface expression and turnover of NMDA receptors could then be analysed by quantitative cell surface labelling techniques such as cell surface biotinylation. Furthermore, electrophysiological analysis of the resulting receptors may identify important changes in NMDA receptor function.

Further characterisation of the properties of native receptors from NR2A^{ΔC} mutant and wild-type mice by radioligand binding studies may clarify the relevance of the *in vitro* findings presented in this thesis. It is clear however, that future investigations into the function of NMDA receptor-interacting proteins require a cross-disciplinary approach to link the biochemical and pharmacological properties of L-glutamate receptor ion channels to the physiology of individual neurons and ultimately to the physiology of the entire animal.

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List of Publications

Abstracts

Rutter A.R., Chazot P.L., Stephenson F.A. (1999) Co-transfection of PSD-95 with cloned NMDA receptor subtypes results in a selective increase in NR2 subunit immunoreactivities. *Biochemical Society Transactions* 27:115.

Rutter A.R., Stephenson F.A. (2000) Coexpression of PSD-95 with NR1/NR2A NMDA receptors decreases the enhancement of [³H]MK801 binding by glutamate. *European Journal of Neuroscience* 12 (suppl. 11) 213.19.

Rutter A.R., Stephenson F.A. (2000) PSD-95 decreases the glutamate sensitivity of [³H]MK801 binding to cloned NR1-1a/NR2A NMDA receptors. *Society for Neuroscience Abstracts* 26, II, 1917.

Research articles

Rutter A.R. and Stephenson F.A. (2000) Coexpression of post-synaptic density 95 protein with NMDA receptors results in enhanced receptor expression together with a decreased sensitivity to L-glutamate. *J. Neurochemistry* 75, 2501-2510

COTRANSFECTION OF PSD-95 WITH CLONED NMDA RECEPTOR SUBTYPES RESULTS IN A SELECTIVE INCREASE IN NR2 SUBUNIT IMMUNOREACTIVITIES

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The N-methyl-D-aspartate subclass of glutamate receptor is a heteromeric ligand-gated cation channel composed of NR1 and NR2 subunits. The C-termini of NR2 subunits are known to interact with membrane-associated guanylate kinases, notably postsynaptic density-95 (PSD-95), thought to cluster NMDA receptors at postsynaptic sites [1]. Here, the effects of coexpression of PSD-95_{c-myc} with NR1a, NR2A, NR1a/NR2A and NR1a/NR2B receptors in human embryonic kidney (HEK) 293 cells were investigated. HEK 293 cells were transfected with NR1a, NR2A, NR1a/NR2A, or NR1a/NR2B in the presence and absence of PSD-95_{c-myc}. The coexpression of PSD-95_{c-myc} with NR1a had no effect on the level of NR1a as determined by quantitative immunoblotting, whether NR1a was expressed alone or in NR1a/NR2 combinations. However when NR2A was expressed alone, PSD-95_{c-myc} induced a 3.5 ± 0.5 -fold ($n = 2$) increase in NR2A immunoreactivity compared to controls. When co-expressed with either NR1a/NR2A or NR1a/NR2B a 3.1 ± 1.2 and a 3.1 ± 1.0 -fold ($n = 6$) increase in NR2 immunoreactivity respectively was induced. [³H] MK801 radioligand binding revealed a 51 ± 17 % ($n = 6$) increase in specific binding to HEK 293 cells transfected with NR1a/NR2A in the presence of PSD-95_{c-myc} compared to controls together with an approximate 3-fold decrease in affinity. Inhibition constants were $K_i = 67.6 \pm 10.3$ nM and 20.1 ± 0.4 nM in the presence and absence of PSD-95_{c-myc} respectively. No change in [³H] MDL105,519 binding was observed. Thus, coexpression of PSD-95_{c-myc} with NR2 subunits results in both an increased level of NR2 subunit and a decreased affinity of the assembled receptor for [³H] MK801.

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COEXPRESSION OF PSD-95 WITH NR1/NR2A NMDA RECEPTORS DECREASES THE ENHANCEMENT OF [³H]MK801 BINDING BY GLUTAMATE

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The N-methyl-D-aspartate (NMDA) subclass of glutamate receptor is a heteromeric ligand-gated cation channel composed of NR1 and NR2 subunits. The C-termini of NR2 subunits are known to interact with the protein, postsynaptic density-95 (PSD-95) which is thought to cluster NMDA receptors at postsynaptic sites. Previously we showed that coexpression of PSD-95 with NR1-1a/NR2A NMDA receptors in human embryonic kidney (HEK) 293 cells resulted in an approximate 3-fold increase in NR2 subunit immunoreactivities and a decrease in the affinity for [³H]MK801(1). Here, we investigated whether the observed decrease in [³H]MK801 affinity was a consequence of a reduced sensitivity to glutamate. EC₅₀ values for the glutamate-dependent enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95 were 8.8 μM and 2.2 μM (mean of n = 2) respectively. The C-terminal ESDV motif of NR2 subunits are thought to be requisite for NR2/PSD-95 interaction. Therefore, truncated NR2A subunits were generated and the effects of coexpression of PSD-95 with NR1-1a/truncated NR2A determined. In contrast with NR1-1a/NR2A wild-type receptors, glutamate enhancement of [³H]MK801 binding to NR1a/truncated-NR2A was not modulated by PSD-95. The decrease in sensitivity to glutamate is therefore dependent upon the presence of the ESDV motif at the C-terminus of the NR2A subunit. The effect of PSD-95 on NR1-1a/truncated NR2A receptors was also investigated by quantitative immunoblotting. These results are in agreement with (2) where it was reported that co-expression of PSD-95 with NR1/NR2B resulted in a decrease in channel sensitivity to glutamate.

(1) Rutter, A. R. *et al.* (1999) Biochemical Society Transactions 27: A115

(2) Yamada, Y. *et al.* (1999) J. Biol. Chem. 274, 6647-6652.

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Abstract Submitted to The Society for Neuroscience

PSD-95 DECREASES THE GLUTAMATE SENSITIVITY OF [³H]MK801 BINDING TO CLONED NR1-1a/NR2A NMDA RECEPTORS

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The N-methyl-D-aspartate (NMDA) subclass of glutamate receptor is a heteromeric ligand-gated cation channel composed of NR1 and NR2 subunits. The C-termini of NR2 subunits are known to interact with the protein, postsynaptic density-95 (PSD-95) which is thought to cluster NMDA receptors at postsynaptic sites. Previously we showed that coexpression of PSD-95 with NR1-1a/NR2A NMDA receptors in human embryonic kidney (HEK) 293 cells resulted in an approximate 3-fold increase in NR2 subunit immunoreactivities with no significant change in the level of NR1-1a $\pm$ PSD-95. An approximate 4-fold decrease in the affinity of NR1-1a/NR2A receptors for [³H]MK801 was also induced by coexpression of PSD-95 (1). Here, we investigated whether the observed decrease in [³H]MK801 affinity was a consequence of a reduced sensitivity to glutamate. EC₅₀ values for the glutamate-dependent enhancement of [³H]MK801 binding to NR1-1a/NR2A receptors in the presence and absence of PSD-95 were 8.9 μ M (2) & 1.8 μ M $\pm$ 0.4 (4) respectively. [³H]MK801 binding to NR1-1a/NR2A^{Trunc} receptors revealed that this approximate 5-fold decrease in glutamate sensitivity induced by PSD-95 was dependent upon the NR2A C-terminal ESDV motif. Further, the PSD-95 induced increase in NR2A immunoreactivities was abolished by deleting the NR2A ESDV motif. These results are in agreement with (2) where it was reported that co-expression of PSD-95 with NR1/NR2B resulted in a decrease in channel sensitivity to glutamate.

(1) Rutter, A.R. et al. (1999) Biochemical Society Transactions 27: A115

(2) Yamada, Y. et al. (1999) J. Biol. Chem. 274, 6647-6652.

Coexpression of Postsynaptic Density-95 Protein with NMDA Receptors Results in Enhanced Receptor Expression Together with a Decreased Sensitivity to L-Glutamate

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Abstract: Coexpression in human embryonic kidney (HEK) 293 cells of the postsynaptic density-95 protein (PSD-95) with NMDA receptor NR2A or NR2B single subunits or NR1-1a/NR2A and NR1-1a/NR2B subunit combinations induced an approximately threefold increase in NR2A and NR2B subunit expression. Deletion of the NR2 C-terminal ESDV motifs resulted in the loss of this increase following coexpression of NR1-1a/NR2A^{Trunc} and NR1-1a/NR2B^{Trunc} with PSD-95. Characterisation of the radioligand binding properties of [³H]MK-801 to NR1-1a/NR2A receptors with or without PSD-95 showed that PSD-95 induced a threefold increase in $B_{\max}$ values and an apparent approximately fivefold decrease in affinity in the presence of 10 μ M L-glutamate. In the presence of 1 mM L-glutamate, the K_i for MK-801 binding to NR1-1a/NR2A with PSD-95 was not significantly different from that for NR1-1a/NR2A without PSD-95. The EC_{50} value for the enhancement of [³H]MK-801 binding by L-glutamate to NR1-1a/NR2A was 1.8 ± 0.4 ($n = 4$) and 8.9 (mean of $n = 2$) μ M in the absence and presence of PSD-95, respectively. Thus, coexpression of PSD-95 with NR1-1a/NR2A results in a decreased sensitivity to L-glutamate and an enhanced expression of NR2A and NR2B subunits. Deletion studies show that this effect is mediated via interaction of the C-terminal ESDV motif of the NR2 subunit with PSD-95. **Key Words:** NMDA receptor—Glutamate—Postsynaptic density-95 protein—MK-801.

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NMDA receptors are a subclass of the excitatory, ionotropic glutamate neurotransmitter receptor family. They are fast-acting cation channels that are activated by the binding of both glutamate and the coagonist glycine, with a high permeability for Ca^{2+} . Six genes encode the NMDA receptor subunits: NR1, NR2A–NR2D, and NR3A. It is thought that native NMDA receptors are formed from the coassembly of two NR1 subunits together with multiple NR2 subunits to form functional receptors with different pharmacological and functional properties (for review, see, e.g., Hollmann and Heinemann, 1994). NMDA receptors play important roles in long-term potentiation; they are pharmacological targets

for intervention postischaemia and, furthermore, recent studies have shown that the actual level of NMDA receptors can have important functional consequences, e.g., overexpression of the NR2B subunit results in enhanced learning and memory in transgenic mice (Tang et al., 1999), and reduced expression of the NR1 subunit yields mice with symptoms characteristic of pharmacologically induced models of schizophrenia (Mohn et al., 1999).

NMDA receptors have been localised to synapses on dendritic spines where they are frequently found in the middle of the synaptic disc (see, e.g., Takumi et al., 1999; Petralia et al., 1994; Racca et al., 2000). At these defined sites, NMDA receptors are associated with a network of proteins including the postsynaptic density-95 protein (PSD-95; alternative nomenclature, SAP90); the presynaptic proteins synaptic-associated protein-97 (alternative nomenclature, hdlg), chapsyn-110/postsynaptic density-93, synaptic-associated protein-102 (reviewed by Sheng, 1996), Shank (Naisbitt et al., 1999), and Yotiao (Lin et al., 1998). The first of these anchoring proteins to be identified was PSD-95 (Kornau et al., 1995). PSD-95 has been shown to interact with the NR2 NMDA receptor subunits and Shaker-type K^+ channel subunits via the amino acid sequence motif E(T/S)XV found at their respective C termini (Kim et al., 1995; Kornau et al., 1995). PSD-95 colocalises with and clusters NMDA receptor subunits following their coexpression in mammalian cells (Kim et al., 1996). Furthermore, mice expressing a C-terminal truncated NR2A transgene, i.e., their NR2A subunits lack the ESDV

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Abbreviations used: HEK, human embryonic kidney; MDL 105,519, (*E*)-3-(2-phenyl-2-carboxyethyl)-4,6-dichloro-1-indole-2-carboxylic acid; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine; PSD-95, postsynaptic density-95 protein; TCP, thienyl-cyclohexylpiperidine.

PSD-95 binding domain, were found to have fewer synaptic NMDA receptors compared with wild-type strains (Steigerwald et al., 2000).

Many studies have characterised both the functional and pharmacological properties of NMDA receptor subtypes following the expression of different subunit combinations in mammalian cells or *Xenopus* oocytes. These host systems do not express the accessory proteins described above. The properties found are not therefore necessarily representative of native synaptic receptors as coassociation with, e.g., PSD-95, may restrict conformational changes within the receptor, or association with other proteins such as kinases, may have important influences on receptor channel activity or receptor pharmacology. Indeed, Yamada et al. (1999) showed recently that coexpression of PSD-95 with the NMDA receptor subunits $\epsilon 2\zeta 1$, i.e., NR2B/NR1, in *Xenopus* oocytes resulted in a decreased sensitivity of the channels to L-glutamate.

In this article, we have used a molecular pharmacological approach to investigate the effect of the coexpression of PSD-95 with both NR1-1a/NR2A and NR1-1a/NR2B cloned receptor subtypes. We provide evidence to support the functional studies of Yamada et al. (1999) in that PSD-95 decreases the sensitivity of NR1-1a/NR2A receptors to L-glutamate. Furthermore, the coexpression of PSD-95 with NR1/NR2 subunits results in elevated expression of the NMDA receptor NR2 subunit.

MATERIALS AND METHODS

Materials

³H-labeled (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid ([³H]MDL 105,519; 74 Ci/mmol), horseradish-linked secondary antibodies, and the ECL Plus western blotting system were from Amersham Pharmacia Biotech Ltd. (Little Chalfont, Bucks, U.K.). (+)-5-[³H]Methyl-10,11-dihydro-5*H*-benzo[*a,d*]cyclohepten-5,10-imine ([³H]MK-801; 28.8 Ci/mmol) was from Du Pont (U.K.) Ltd. (Stevenage, Hertfordshire, U.K.). MK-801 was from Research Biochemicals International (Natick, MA, U.S.A.). Anti-NR1 911–920 (anti-NR1 C2), anti-NR2A 1,381–1,394, anti-NR2A/2B 1,435–1,445, and anti-NR2B 46–60 Cys antibodies were prepared and characterised as previously described (Chazot et al., 1994; Chazot and Stephenson, 1997*a,b*). Anti-c-Myc 9E10 mouse monoclonal antibodies were purchased from Sigma-Aldrich (Dorset, U.K.). The Quick Change Site-Directed Mutagenesis kit was from Stratagene (La Jolla, CA, U.S.A.). The CytoTox 96 kit was from Promega (Madison, WI, U.S.A.). NMDA receptor cDNAs were generous gifts from Professors S. Nakanishi (Kyoto, Japan) and M. Mishina (Nigata, Japan); pGW1PSD-95 and pGW1PSD95_{c-Myc} were generous gifts from Dr. Morgan Sheng (Boston, MA, U.S.A.). The c-Myc tag sequence, EQKLISEEDL, was inserted at amino acid 12 of PSD-95. All other materials were obtained from commercial sources.

Mammalian cell transfections and cell homogenate preparation

Human embryonic kidney (HEK) 293 cells were cultured and transfected with pCISNR1-1a, pCISNR2A, pCISNR1-1a/

NR2A, or pCISNR1-1a/NR2B cDNA clones in the presence and absence of either pGW1PSD-95_{c-Myc} or pGW1PSD-95 using the calcium phosphate method as previously described (Cik et al., 1993). All transfections used a total of 20 μ g of DNA. NMDA receptor:PSD-95 clones were transfected using a 1:1 ratio; NR1-1a:NR2 binary combinations used a 1:3 ratio. Thus, a triple transfection used 2.5 μ g of pCISNR1-1a:7.5 μ g of pCISNR2:10 μ g of pGW1PSD-95. Where appropriate, pSV β -galactosidase or pCIS was included in the transfection mixture. Cells were cultured posttransfection in the presence of 1 mM ketamine where necessary to prevent NMDA receptor-mediated cytotoxicity. Cells were harvested 24–36 h posttransfection, and homogenates were prepared, adjusted to 0.5 mg of protein/ml, and analysed by quantitative immunoblotting (Chazot et al., 1992; Hawkins et al., 1999). For [³H]MK-801 and [³H]MDL 105,519 radioligand binding studies, cell homogenates were prepared as previously described (Chazot et al., 1992, 1998). For the glutamate enhancement of [³H]MK-801 radioligand binding studies, cell homogenates were washed extensively to remove endogenous glutamate. Thus, HEK 293 cells were collected by centrifugation (800 *g* for 5 min at 4°C) and homogenised in ice-cold 50 mM Tris-citrate (pH 7.1) containing 5 mM EDTA and 5 mM EGTA (buffer 1) using a Dounce (glass/glass) homogeniser (50 strokes). The homogenate was processed by five cycles of centrifugation (120,000 *g* for 30 min at 4°C) and homogenisation (50 strokes) with final resuspension in buffer 1 to a protein concentration of ~1.5 mg/ml. For all studies, variation in transfection efficiency between flasks was minimized by pooling cell homogenates from three individually transfected flasks.

Immunoblotting

Immunoblotting was performed as previously described (Duggan et al., 1991). Protein samples (25–50 μ g) were precipitated using the chloroform/methanol method, and sodium dodecyl sulphate–polyacrylamide gel electrophoresis was carried out under reducing conditions in 7.5% polyacrylamide slab minigels. Affinity-purified anti-NR1 (C2 exon), anti-NR2A (1,381–1,394), anti-NR2A/2B (1,435–1,445), and anti-NR2B (46–60) rabbit antibodies were used at final concentrations of 1–5 μ g/ml. Anti-c-Myc 9E10 mouse monoclonal antibodies were used at a dilution of 1:2,000. Rabbit and mouse immunoglobulin horseradish-linked whole antibodies were used at a final dilution of 1:2,000. Immunoreactivities were detected using the ECL Plus western blotting detection system. Immunoreactive bands were scanned and quantified by molecular densitometry using a Molecular Dynamics ImageQuant in the linear range of the film. For the quantitative comparative studies, equal amounts of protein were applied per gel lane. Both the protein concentration and the primary antibody concentrations were adjusted to establish the linear range of the detection system (Chazot and Stephenson, 1997*b*).

Radioligand binding assays

[³H]MDL 105,519 binding assays were performed as previously described (Chazot et al., 1998) using polyethylenimine filtration with 100 μ M glycine for the determination of non-specific binding and an incubation time of 90 min at 4°C. [³H]MK-801 saturation and displacement assays to transfected cell homogenates (50 μ g of protein) were performed in the presence of either 10 μ M or 1 mM L-glutamate and 30 μ M glycine using 100 μ M thienylcyclohexylpiperidine (TCP) for the determination of nonspecific binding, an incubation time of 2 h at 22°C, and polyethylenimine filtration for the separation of bound and free radioligand (Chazot et al., 1994). All

[³H]MK-801 binding experiments were carried out to cells transfected with NR1-1a/NR2A clones because [³H]MK-801 binding to NR1-1a/NR2B cells yielded low signal:noise ratios. This may be due to the reported three- to fivefold lower peak open probability for NR1A/NR2B compared with NR1A/NR2A (Chen et al., 1999). For the dose-dependent glutamate enhancement of [³H]MK-801 binding studies, well-washed transfected cell homogenates (150 μ g of protein) were incubated with 2 nM [³H]MK-801 and 30 μ M glycine for 2 h at 22°C in the presence of a 0–10 mM range of glutamate concentration. Separation of bound and free radioligand was again by polyethylenimine filtration. Binding curves were analysed by the Inplot program (GraphPad Software; Prof. M. A. Simmonds, School of Pharmacy, London, U.K.). Note that experiments comparing the radioligand binding properties to a defined receptor in the presence and absence of PSD-95 were always carried out in parallel.

Synthesis of NR2A and NR2B C-terminal truncation mutants, NR2A^{Trunc} and NR2B^{Trunc}

The ESDV C-terminal motifs of the NR2A and NR2B subunits were deleted by mutagenesis. A stop codon was introduced converting a G→T 12 bases from the 3' end of both clones. The following complementary oligonucleotides were used to induce single base changes: NR2A, 5'-GATCTTAGACATCA-GATTAAATACTAGGCATTTCTTGTACACACG-3' + complementary oligonucleotide; NR2B, 5'-GACATCAGACTA-AATACTAGAAAG-3' + complementary oligonucleotide. Mutagenesis was carried out using the Stratagene Quick Change Site-Directed Mutagenesis kit according to the manufacturer's instructions. The mutations were verified by nucleotide sequencing using the ABI 310 Automated Genetic Analyser. Quantitative immunoblotting of HEK 293 cells transfected with either pCIS NR1-1a/pCISNR2A or pCISNR1-1a/pCISNR2A^{Trunc} revealed that the wild-type and truncated NR2A subunits (M_r of both = 180,000) were expressed to similar levels. Similarly, HEK 293 cells cotransfected with either pCISNR1-1a/pCISNR2B or pCISNR1-1a/pCISNR2B^{Trunc} revealed similar levels of expression of the NR2B subunits when the immunoblots were analysed using anti-NR2B (46–60) antibodies. Anti-NR2A/B (1,435–1,445) antibodies did not detect the M_r 180,000 NR2B subunit because the peptide used to generate the antibody, YKKMP-SIESDV, contains the C-terminal truncated four amino acids. HEK 293 cells transfected with either NR1-1a/NR2A or NR1-1a/NR2A^{Trunc} displayed similar affinities for [³H]MK-801 in the presence of 1 mM glutamate, i.e., with dissociation constant (K_D) values of 12 ± 7 (n = 5) and 13 ± 0.6 nM (n = 3), respectively. Furthermore, transfection of HEK 293 cells with NR1-1a/NR2A^{Trunc} or NR1-1a/NR2B^{Trunc} resulted in 23 and 23% (n = 2) receptor-mediated cell death, respectively, consistent with the levels of cytotoxicity induced by the corresponding wild-type receptors when cultured posttransfection in the absence of 1 mM ketamine. Thus, for the three parameters assayed, the NR2^{Trunc} mutants behaved as wild-type NR2 subunits with respect to their ability to express, to associate with NR1-1a subunits to form functional NMDA receptors, and to bind [³H]MK-801 with high affinity.

Cytotoxicity assays

HEK 293 cells were transfected with NMDA receptor clones in the presence and absence of pGWIWPSD-95_{c-Myc} and cultured in ketamine-free medium. At 20 h posttransfection the percent cell death was determined using the Promega CytoTox 96 cytotoxicity assay according to the manufacturer's instructions.

Protein determination

Protein concentrations were determined using the method of Lowry et al. (1951) with bovine serum albumin as the standard protein.

RESULTS

Effect of PSD-95_{c-Myc} on expression levels of NMDA receptor subunits in HEK 293 cells following cotransfection of pGIWPSD-95_{c-Myc} with either NR1-1a or NR2 single subunits or NR1-1a/NR2 binary combinations

Initial experiments expressed NR1-1a/NR2A and NR1-1a/NR2B receptors in the presence and absence of PSD-95_{c-Myc} in HEK 293 cells. The resultant cell homogenates were analysed by quantitative immunoblotting using anti-NR1 (911–920), anti-NR2A (1,381–1,394), anti-NR2B (46–60) Cys, anti-NR2A (1,435–1,445), and anti-c-Myc antibodies. Representative results are shown in Fig. 1. It was found that coexpression of PSD-95_{c-Myc} with NR1-1a/NR2A and NR1-1a/NR2B resulted in a 2.9 ± 1.1 - (n = 18; $p < 0.001$ by t test) and a 3.4 ± 1.4 -fold (n = 8; $p < 0.01$ by t test) increase in NR2A and NR2B subunit immunoreactivities, respectively (Fig. 1A, B, and E) with no significant change in the expression level of the NR1-1a subunit. The ratio of NR1-1a subunit immunoreactivities in the presence and absence of PSD-95_{c-Myc} was 1.0 ± 0.2 (n = 20) for both NR1-1a/NR2A and NR1-1a/NR2B. The same results were observed when PSD-95_{c-Myc} was expressed with either the NR1-1a or NR2A subunits alone, i.e., a 3.2 ± 1.5 -fold (n = 3) increase in NR2A but no significant change in the expression level of the NR1-1a subunit.

The ESDV motif of the NR2A and NR2B C-termini is known to be necessary for the association between PSD-95 and the NR2 subunits (Bassand et al., 1999). Therefore, to assess whether the specific increase in NR2 immunoreactivities was dependent on an interaction between PSD-95_{c-Myc} and the C-terminal tails of NR2 subunits, PSD-95_{c-Myc} was coexpressed with NR1-1a/NR2A^{Trunc} and NR1-1a/NR2B^{Trunc}. Representative results are shown in Fig. 1C and D. Consistent with previous experiments, PSD-95_{c-Myc} induced an approximately threefold increase in wild-type NR2A and NR2B immunoreactivities with no significant change in the level of NR1-1a. However, this threefold increase induced by PSD-95_{c-Myc} was not seen in experiments where the NR1-1a/NR2^{Trunc} subunits were coexpressed. Fold changes in the presence of PSD-95_{c-Myc} were 0.75 ± 0.2 (n = 6) for NR2A^{Trunc} and 0.6 (n = 2) for NR2B^{Trunc}. Thus, the deletion of the COOH-terminal motif abolished the increase in NR2A and NR2B immunoreactivities that was induced by PSD-95_{c-Myc}.

It is interesting that in the absence of PSD-95, anti-NR2A (1,381–1,394) antibodies sometimes detected a doublet with M_r 180,000 and M_r 175,000 (see, e.g., Fig. 1C, lane NR2A, – T); in those immunoblots where the lower-molecular-weight species was detectable, in the

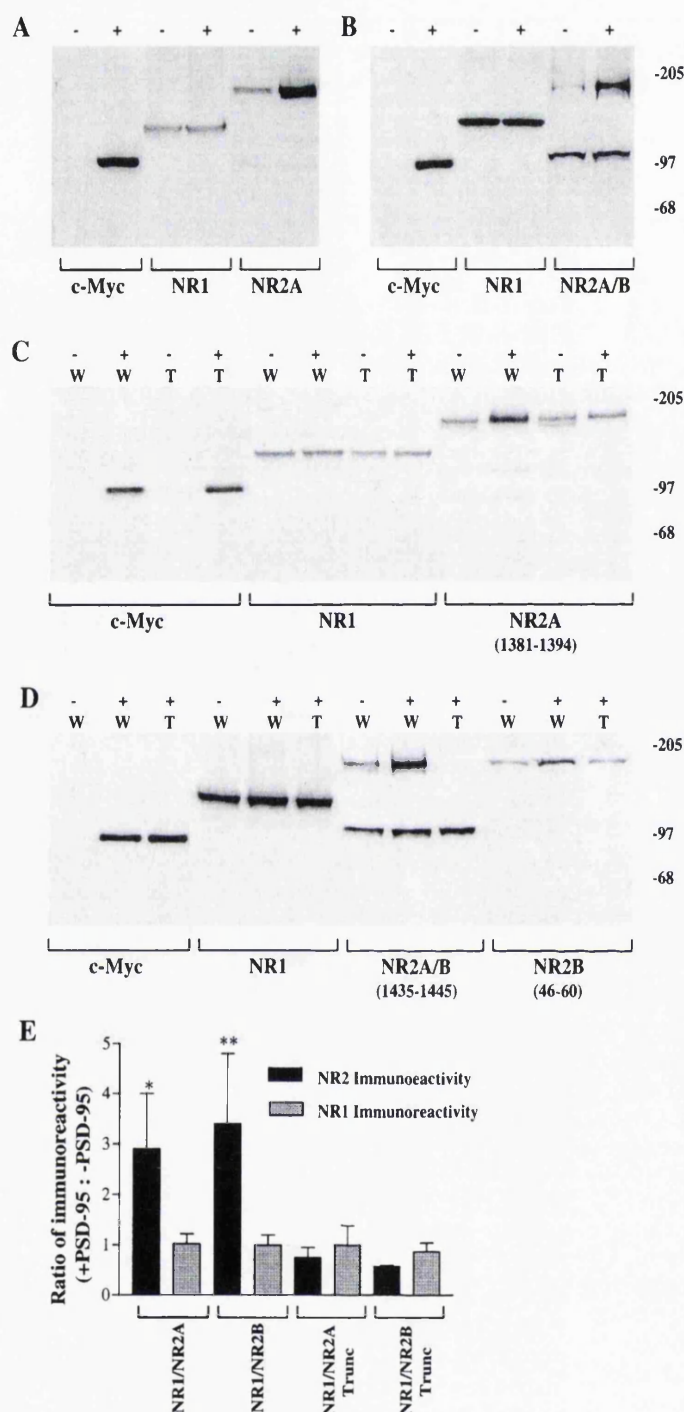


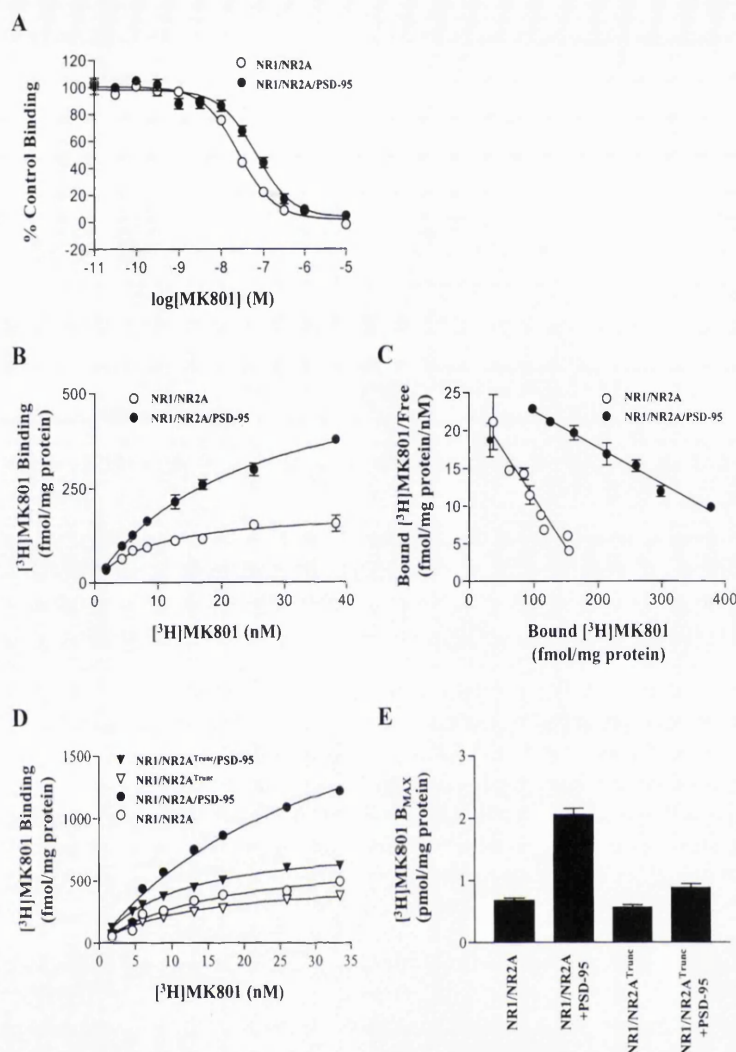
FIG. 1. Effect of coexpression of PSD-95_{c-Myc} in HEK 293 cells on the level of NMDA receptor subunits determined by immunoblotting. HEK 293 cells were transfected with NMDA receptor NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2A^{Trunc}, or NR1-1a/NR2B^{Trunc} clones in the presence and absence of PSD-95_{c-Myc}. Cells were harvested and analysed by quantitative immunoblotting using 25 µg of protein per gel lane as described in Materials and Methods. **A:** HEK 293 cells transfected with NR1-1a/NR2A in the presence (+) and absence (-) of PSD-95_{c-Myc} using the antibodies anti-c-Myc, anti-NR1 (911-920), and anti-NR2A (1,381-1,394). **B:** HEK 293 cells transfected with NR1-1a/NR2B in the presence (+) and absence (-) of PSD-95_{c-Myc} using the antibodies anti-c-Myc, anti-NR1 (911-920), and anti-NR2A/B (1,435-1,445). **C:** HEK 293 cells transfected with wild-type (W) NR1-1a/NR2A or mutant (T) NR1-1a/NR2A^{Trunc} in the presence (+) and absence (-) of PSD-95_{c-Myc} using the antibodies anti-c-Myc, anti-NR1 (911-920), and anti-NR2A (1,381-1,394). **D:** HEK 293 cells transfected with NR1-1a/NR2B (W) in the presence (+) and absence (-) of PSD-95_{c-Myc} or NR1-1a/NR2B^{Trunc} (T) in the presence (+) of PSD-95_{c-Myc} using the antibodies anti-c-Myc, anti-NR1 (911-920), anti-NR2A/B (1,435-1,445), and anti-NR2B (46-60). In A-D, the positions of protein standards are shown on the right. **E:** Histogram summarises the results of the quantitative immunoblotting. Results are expressed as the ratio of immunoreactive intensities measured in the presence of PSD-95_{c-Myc} to intensities measured in the absence of PSD-95_{c-Myc}. Data are mean ± SD (bars) values for at least n = 4 separate transfections with n = 3 immunoblots per transfection. *t* test, **p* < 0.001, ***p* < 0.01 by *t* test.

presence of PSD-95, only a single M_r immunoreactive species was detected (Fig. 1C, lane NR2A, + T). This suggests that PSD-95 may protect the NR2A subunit against proteolytic degradation. [Note that anti-PSD-95 antibodies showed no immunoreactivity in untransfected cells (data not shown); also, the M_r 100,000 species recognised by the anti-NR2A/B 1435-1445 antibody is a nonspecific protein because it is present in both the untransfected and transfected cells.]

Effect of coexpression of PSD-95_{c-Myc} on [³H]MDL 105,519 and [³H]MK-801 radioligand binding properties of NR1-1a/NR2A receptors assayed under standard conditions

Radioligand binding of the NMDA receptor glycine site antagonist [³H]MDL 105,519 was carried out to cell homogenates transfected with NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc}. Saturation analyses revealed that cotransfection with PSD-95_{c-Myc} did not

FIG. 2. Effect of coexpression of PSD-95_{c-Myc} in HEK 293 cells on [³H]MK-801 radioligand binding to NR1-1a/2A and NR1-1a/NR2A^{Trunc} receptors. HEK 293 cells were transfected with NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Cell homogenates were prepared and assayed for [³H]MK-801 radioligand binding in the presence of 10 μ M glutamate and 30 μ M glycine as described in Materials and Methods. **A:** Displacement of [³H]MK-801 by MK-801 from HEK 293 cells transfected with NR1-1a/NR2A in the presence (●) and absence (○) of PSD-95_{c-Myc}. Results are expressed as a percentage of control [³H]MK-801 specific binding. Data are mean $\pm$ SD (bars) values for triplicate determinations for $n = 5$ independent transfections. Analysis of the curve by nonlinear regression (GraphPad Prism) resolved a single binding site in both the presence and absence of PSD-95_{c-Myc} with K_i values summarised in Table 1. **B and C:** A representative saturation curve (B) and Scatchard transformation (C) for the specific binding of [³H]MK-801 to HEK 293 cells transfected with NR1-1a/NR2A in the presence (●) and absence (○) of PSD-95_{c-Myc}. Analysis of the curves by linear regression resolved single, high-affinity binding sites in the presence and absence of PSD-95_{c-Myc} with binding parameters summarised in Table 1. Data are mean $\pm$ SD (bars) values for $n = 3$ determinations for at least $n = 2$ independent transfections. **D:** Saturation curve for the specific binding of [³H]MK-801 in the presence of 1 mM L-glutamate to HEK 293 cells transfected with NR1-1a/NR2A or NR1-1a/NR2A^{Trunc} in the presence (solid symbols) and absence (open symbols) of PSD-95_{c-Myc}. Data are mean $\pm$ SD (bars) values for triplicate determinations. Curves are representative of a typical experiment replicated twice. **E:** Histogram summarises the B_{max} values for the binding of [³H]MK-801 in the presence of 1 mM L-glutamate to NR1-1a/NR2A wild-type and truncated (Trunc) receptors in the presence and absence of PSD-95_{c-Myc}. Values are shown in Table 1.



change the binding parameters for [³H]MDL 105,519. The K_D values for direct [³H]MDL 105,519 binding to NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc}, respectively, were 3.3 ($n = 2$) and 3.5 nM ($n = 2$) with corresponding B_{max} values of 4.2 and 4.2 pmol of [³H]MDL 105,519 binding sites/mg of protein (both, mean of $n = 2$; data not shown).

In contrast, coexpression of PSD-95_{c-Myc} with NR1-1a/NR2A had a significant effect on the binding properties of the NMDA receptor noncompetitive antagonist [³H]MK-801. The results are shown in Fig. 2. [³H]MK-801 displacement studies found that PSD-95_{c-Myc} induced a 4.9 ± 1.6 -fold decrease in the affinity of NR1-1a/NR2A receptors for [³H]MK-801. Values for the apparent K_i values for displacement of [³H]MK-801 by MK-801 from cells transfected with NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc} were 59 ± 19 ($n = 5$) and 12 ± 7 nM ($n = 5$), respectively. Full

saturation analyses revealed a similar change in K_D with a concomitant approximately threefold increase in B_{max} . K_D values for [³H]MK-801 binding to NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc} were 25 ($n = 2$) and 9 ($n = 2$) nM with B_{max} values of 0.8 ($n = 2$) and 0.2 ($n = 2$) pmol of [³H]MK-801 binding sites/mg of protein, respectively (Table 1). [³H]MK-801 binding to cell homogenates prepared from HEK 293 cells transfected with NR1-1a/NR2A^{Trunc} with or without PSD-95_{c-Myc} yielded binding parameters of $K_D = 13 \pm 1$ nM and $B_{max} = 0.6 \pm 0.1$ pmol/mg of protein without PSD-95_{c-Myc} ($n = 3$) compared with $K_D = 12 \pm 1$ nM ($n = 3$) and $B_{max} = 0.9 \pm 0.1$ pmol/mg of protein ($n = 3$) with PSD-95_{c-Myc} ($n = 3$) (Fig. 2D and E and Table 1). Thus, the fold increase in the number of [³H]MK-801 binding sites induced by PSD-95 is dependent on the presence of the C-terminal ESDV motif (Fig. 2E).

TABLE 1. Summary of parameters for binding of [^{3} H]MK-801 to wild-type and mutant NR1-1a/NR2A^{Trunc} receptors in the presence of 1 mM and 10 μ M L-glutamate

Transfection conditions	L-Glutamate	K_i (nM)	K_D (nM)	B_{max} (pmol/mg of protein) ^a	EC ₅₀ (μ M)
NR1-1a/NR2A					
– PSD-95	10 μ M	12 $\pm$ 7 (5)	9 (2)	0.2 (2)	1.8 $\pm$ 0.4 (4)
+ PSD-95	10 μ M	59 $\pm$ 19 (5)	25 (2)	0.8 (2)	8.9 (2)
– PSD-95	1 mM	12 (2)	13 (2)	0.7 (2)	—
+ PSD-95	1 mM	13 $\pm$ 9 (3)	22 (2)	2.1 (2)	—
NR1-1a/NR2A ^{Trunc}					
– PSD-95	1 mM	ND	13 $\pm$ 1 (3)	0.6 $\pm$ 0.1 (3)	1.5 (1)
+ PSD-95	1 mM	ND	12 $\pm$ 1 (3)	0.9 $\pm$ 0.1 (3)	—

The L-glutamate concentration is that used in the [^{3} H]MK-801 radioligand binding assays. K_i is the inhibitory constant for MK-801 determined by displacement assay. K_D and B_{max} are the dissociation constant and the number of binding sites for [^{3} H]MK-801 determined by direct binding, respectively. EC₅₀ is the concentration of L-glutamate that elicits 50% maximal enhancement of specific [^{3} H]MK-801 binding. Data are either mean $\pm$ SD values for *n* determinations or the mean of *n* = 2 values.

^a Differences in the absolute B_{max} values are probably a reflection of transfection efficiency; importantly, the fold difference with and without PSD-95 is of similar magnitude between different sets of experiments.

Effects of coexpression of PSD-95_{c-Myc} on glutamate sensitivity of [^{3} H]MK-801 binding to NR1-1a/NR2A receptors expressed in HEK 293 cells

MK-801 is an open channel, noncompetitive antagonist of the NMDA receptor. [^{3} H]MK-801 radioligand binding is enhanced by L-glutamate in a dose-dependent manner (Ransom and Stec, 1988). Standard conditions in our laboratory have used saturating concentrations, i.e., 10 μ M glutamate, in all tubes to allow access of the [^{3} H]MK-801 radioligand for its binding site (Chazot et al., 1992). A reduced K_D for MK-801 may therefore be a result of a decreased affinity of NR1-1a/NR2A receptors for glutamate. To test this hypothesis, [^{3} H]MK-801 binding at a concentration of 2 nM was measured in the presence of increasing concentrations of glutamate (0–10 mM) to NR1-1a/NR2A receptors expressed in the presence and absence of PSD-95_{c-Myc}. The results are shown in Fig. 3. In both cell homogenate preparations, glutamate caused a dose-dependent increase in [^{3} H]MK-801 radioligand binding with or without PSD-95_{c-Myc}. The concentrations that elicited 50% of the maximal enhancement, i.e., the EC₅₀ values were 8.9 (*n* = 2) and 1.8 $\pm$ 0.4 μ M (*n* = 4) in the presence and absence of PSD-95_{c-Myc}, respectively. Thus, coexpression of PSD-95_{c-Myc} with NR1-1a/NR2A results in an approximately fivefold decrease in sensitivity of the enhancement of [^{3} H]MK-801 binding to glutamate. To confirm that the decrease in glutamate sensitivity was dependent on an interaction between the NR2A subunit and PSD-95_{c-Myc}, the enhancement of [^{3} H]MK-801 binding by L-glutamate to NR1-1a/NR2A^{Trunc} with or without PSD-95 was studied. The EC₅₀ for the L-glutamate stimulation of [^{3} H]MK-801 binding to NR1-1a/NR2A^{Trunc} was not significantly different from the value found for NR1-1a/NR2A, i.e., 1.5 μ M. [^{3} H]MK-801 binding was thus determined at 10 nM, 10 μ M, and 1 mM glutamate to NR1-1a/NR2A and NR1-1a/NR2A^{Trunc} in the presence and absence of PSD-95_{c-Myc} (Fig. 3B). The ratios of the percent enhancement of [^{3} H]MK-801 specific binding at

1 mM:10 μ M glutamate to wild-type NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc} were 1.7 $\pm$ 0.2 (*n* = 4) and 1.0 $\pm$ 0.1 (*n* = 5), respectively. The ratios of the percent enhancement of [^{3} H]MK-801 specific binding at 1 mM:10 μ M glutamate to NR1-1a/NR2A^{Trunc} receptors in the presence and absence of PSD-95_{c-Myc} were 1.1 $\pm$ 0.1 (*n* = 4) and 1.1 $\pm$ 0.02 (*n* = 4), respectively. Thus, truncation of the C-terminal ESDV amino acids of the NR2A subunit abolished the decreased ability of glutamate to enhance [^{3} H]MK-801 binding to NR1-1a/NR2A receptors induced by PSD-95_{c-Myc}.

Effect of glutamate concentration on affinity of [^{3} H]MK-801 binding to NR1-1a/NR2A expressed in the presence and absence of PSD-95_{c-Myc} in HEK 293 cells

Because PSD-95_{c-Myc} induced a decrease in the EC₅₀ for the enhancement of [^{3} H]MK-801 specific binding by glutamate (see above) and because the K_D for [^{3} H]MK-801 was determined in the presence of 10 μ M glutamate, it was possible that this concentration was not optimal and may thus explain the observed decrease in affinity. Thus, [^{3} H]MK-801 displacement assays to NR1-1a/NR2A receptors expressed in the presence and absence of PSD-95_{c-Myc} were repeated but now in the presence of 1 mM glutamate. The K_i values for the displacement of [^{3} H]MK-801 by MK-801 from NR1-1a/NR2A receptors were 59 $\pm$ 19 (*n* = 5) and 13 $\pm$ 9 nM (*n* = 3) in the presence of PSD-95_{c-Myc} at 10 μ M and 1 mM glutamate, respectively, and 12 $\pm$ 7 (*n* = 5) and 12 nM (*n* = 2), respectively, in the absence of PSD-95_{c-Myc} (Fig. 4 and Table 1). Thus, by increasing the glutamate concentration to 1 mM, the affinity of NR1-1a/NR2A receptors for MK-801 in the presence of PSD-95_{c-Myc} was increased to a value comparable to that of NR1-1a/NR2A receptors in the absence of PSD-95_{c-Myc}.

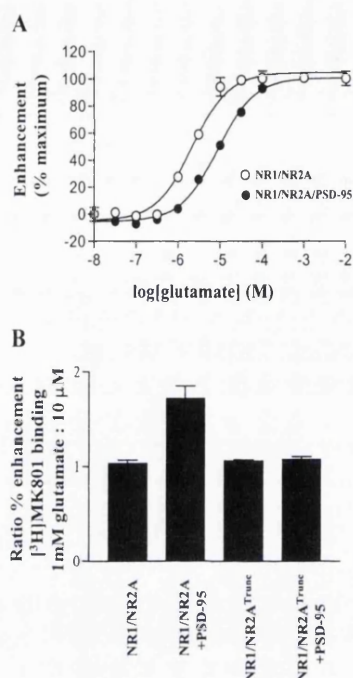


FIG. 3. Effect of PSD-95_{c-Myc} on L-glutamate sensitivity of [³H]MK-801 binding to HEK 293 cells transfected with NR1-1a/NR2A or NR1-1a/NR2A^{Trunc} receptor clones. HEK 293 cells were transfected with NR1-1a/NR2A or NR1-1a/NR2A^{Trunc} NMDA receptor clones in the presence and absence of PSD-95_{c-Myc}. Cells were harvested and washed extensively, and the homogenates (150 μg of protein) were assayed for [³H]MK-801 (2 nM) binding in the presence of 30 μM glycine and increasing concentrations of L-glutamate in the range 0–10 mM. **A:** Enhancement of [³H]MK-801 binding to NR1-1a/NR2A receptors by glutamate in the presence (●) and absence (○) of PSD-95_{c-Myc}. The enhancement of [³H]MK-801 specific binding by glutamate at each point was calculated as the percentage of the control, specific binding, i.e., in the absence of added L-glutamate. It is expressed here as a percentage of the maximal enhancement induced by 10 mM glutamate. Data are mean ± SD (bars) values for n = 3 determinations for n = 2 independent transfections. Dose-response curves were analysed by GraphPad Prism, and the EC₅₀ values are summarised in Table 1. Note that the maximal percent enhancement by 1 mM L-glutamate was 207 ± 62 (n = 4) and 221% (mean of two determinations) in the absence and presence of PSD-95, respectively. **B:** Enhancement of [³H]MK-801 binding to NR1-1a/NR2A and NR1-1a/NR2A^{Trunc} receptors by glutamate in the presence and absence of PSD-95_{c-Myc}. HEK 293 cells were transfected with NR1-1a/NR2A or NR1-1a/NR2A^{Trunc} in the presence and absence of PSD-95_{c-Myc}, and cell homogenates were prepared with extensive washing and assayed for [³H]MK-801 specific binding in the presence of 30 μM glycine and 10 nM, 10 μM, or 1 mM glutamate. The enhancement of [³H]MK-801 binding by glutamate was calculated as for A. Results are presented as the ratios of the percent enhancement at 1 mM glutamate to the percent enhancement at 10 μM L-glutamate. Triplicate determinations were carried out for each point for at least n = 3 independent transfections. Data are mean ± SD (bars) values.

fection in the presence of an NMDA receptor antagonist; 1 mM ketamine is routinely used. The effect of coexpression of PSD-95 with NR1-1a/NR2A and NR1-1a/NR2B on cell cytotoxicity was therefore studied. The expression of PSD-95 alone did not result in significant cell death compared with HEK 293 cells transfected with control pCIS. HEK 293 cells transfected with pCISNR1-1a/pCISNR2A with or without PSD-95 or pCISNR1-1a/pCISNR2B with or without PSD-95 resulted in 20, 22, 26, and 25% cell death, respectively, compared with HEK 293 cells cultured posttransfection in the presence of 1 mM ketamine (mean of n = 2 determinations). The inclusion of pGW1PSD-95_{c-Myc} in the transfection mixture therefore had no significant effect on cell death.

DISCUSSION

PSD-95 is an abundant component of the postsynaptic densities of mammalian brain. It is known to interact with the COOH-terminal ESDV motifs of NMDA receptor NR2 subunits (Kornau et al., 1995; Kim et al., 1996; Niethammer et al., 1996; Bassand et al., 1999) and other postsynaptic proteins, including neuronal nitric oxide

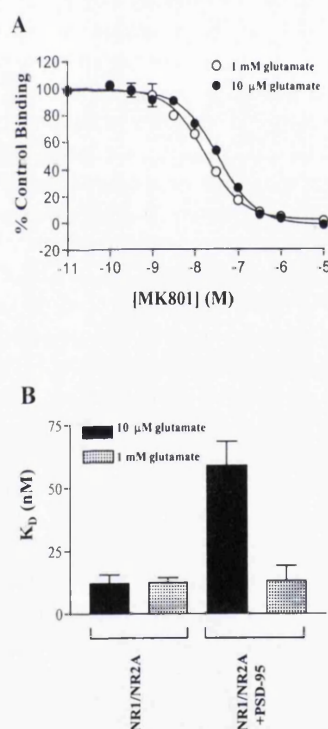


FIG. 4. Effect of elevated L-glutamate concentrations on affinity of NR1-1a/NR2A receptors for [³H]MK-801 in the presence and absence of PSD-95_{c-Myc}. HEK 293 cells were transfected with NR1-1a/NR2A in the presence and absence of PSD-95_{c-Myc}, and cell homogenates were prepared and assayed for [³H]MK-801 binding in the presence of 30 μM glycine and either 1 mM (○) or 10 μM (●) L-glutamate, all as described in Materials and Methods. **A:** Displacement of [³H]MK-801 specific binding by MK-801 to NR1-1a/NR2A receptors. **B:** Histogram shows the effect of L-glutamate concentration on the K_d for the binding of MK-801 to NR1-1a/NR2A receptors in the presence and absence of PSD-95_{c-Myc}. Data are mean ± SD (bars) values.

Effect of coexpression of PSD-95 with NR1-1a/NR2A in HEK 293 cells on cytotoxicity posttransfection

The expression of NR1-1a/NR2A or NR1-1a/NR2B in HEK 293 cells results in cell death. This cytotoxicity can be prevented by culturing the HEK 293 cells posttrans-

synthase (Sattler et al., 1999), guanylate kinase-associated protein (GKAP) (Kim et al., 1997; Yamada et al., 1999), p135 SynGAP (Chen et al., 1998; Kim et al., 1998), and Shaker-type K^+ channels (reviewed by Sheng, 1996). Coexpression of PSD-95 with NMDA receptors in COS cells induces receptor clustering (Kim et al., 1996; Hsueh et al., 1997). More recently PSD-95 has been implicated in mechanisms of long-term potentiation and in forms of learning and memory (Migaud et al., 1998). Few investigations, however, have been made into the direct consequences of the association of PSD-95 with NMDA receptors. In this article, we have described results showing that the interaction of PSD-95 with NMDA receptors does indeed induce changes in both receptor function and NMDA receptor expression.

Effect of coexpression of PSD-95 with NR1-1a/NR2A NMDA receptors on the level of NR1-1a and NR2 subunit immunoreactivities

Initial experiments showed that coexpression of PSD-95 with NR2A or NR2B expressed alone or in NR1-1a/NR2 combinations resulted in an approximately threefold increase in NR2A and NR2B immunoreactivities. PSD-95, however, did not induce any changes in NR1 immunoreactivity whether NR1-1a was expressed alone or in NR1-1a/NR2A subunit combinations. This increased NR2 subunit expression was not apparent when the truncated NR2A and NR2B subunits were coexpressed with PSD-95. The truncated mutants lack their C-terminal ESDV motifs, the amino acid sequence that has been shown to be essential for NR2:PSD-95 interaction, thus suggesting that the increased level of expression is dependent on an interaction between PSD-95 and the NR2 subunits. In similar experiments, Yamada et al. (1999) reported that coexpression of $\epsilon 2\zeta 1$ (NR1NR2B) subunits plus PSD-95 in *Xenopus* oocytes resulted in an increase in levels of not only NR2 subunits but also NR1 compared with expression levels in the absence of PSD-95. The ratio of NR1:NR2 subunit immunoreactivities was the same in both expression conditions, thus implying an increase in cell surface receptors. Similarly, Thomas and Westbrook (1999) reported an enhanced cell surface expression of NMDA receptors as determined by the increase in the peak amplitude of current generated by NR1-1a/NR2A with PSD-95 compared with controls. It was suggested that this may be due to a decreased turnover of receptor (Thomas and Westbrook, 1999). An increase in K^+ channel-mediated currents in HEK 293 cells transfected with the K^+ channel clones, Kir4.1, and PSD-95 has also been reported (Horio et al., 1997), again suggesting an increased level of cell surface expression of Kir4.1 channels. In a more recent study, coexpression of a different K^+ channel, Kv1.4, with PSD-95 did not result in an increased level of Kv1.4 (Jugloff et al., 2000). The presence of PSD-95 did, however, slow the internalization rate of the expressed Kv1.4 potassium channels (Jugloff et al., 2000). It is thus proposed that PSD-95 acts to stabilise the receptor/ K^+ channel in the postsynaptic membrane, per-

haps by reducing the turnover time of NMDA receptors or K^+ channel subunits. Alternatively, PSD-95 may protect NMDA receptor subunits from proteolytic degradation within the cell. Indeed, in some immunoblots, e.g., Fig. 1, it can be seen that PSD-95 prevents the detection of lower-molecular-weight immunoreactive bands, which are probably clipped forms of both the NR1-1a and NR2A subunits.

Effect of coexpression of PSD-95_{c-Myc} with NR1-1a/NR2 NMDA receptors on binding properties of [³H]MK-801 and [³H]MDL 105,519

The parameters for the radioligand binding of [³H]MDL 105,519 to cell homogenates prepared from HEK 293 cells transfected with NR1-1a/NR2A with or without PSD-95_{c-Myc} were not significantly different. Because [³H]MDL 105,519 binds with equal affinity to assembled and unassembled NR1-1a subunits (Chazot et al., 1998), this is in accord with the quantitative immunoblotting results, i.e., no change in the expression level of NR1-1a with or without PSD-95_{c-Myc}. In contrast, saturation analyses performed with [³H]MK-801 and assayed in the presence of 10 μ M glutamate revealed an approximately threefold increase in $B_{\max}$ values for NR1-1a/NR2A receptors coexpressed with PSD-95 together with an apparent approximately fivefold decrease in affinity. When NR1-1a/NR2A^{Trunc} receptors were coexpressed with PSD-95_{c-Myc}, the fold increase in $B_{\max}$ was significantly reduced and approached control values. These results substantiate the fact that the observed changes are a result of the interaction between PSD-95 and the NR2A subunit. Furthermore, they are in agreement with the approximately threefold increase in the NR2 subunit immunoreactivities seen in the presence of PSD-95. However, [³H]MK-801 binds with high affinity to binary NR1-1a/NR2A or NR1-1a/NR2B receptors; thus, because the number of [³H]MK-801 binding sites is increased in the presence of PSD-95, one would predict that there should be a parallel increase in *both* NR1-1a and NR2A subunit immunoreactivities. A possible explanation for this disparity is that in HEK 293 cells transfected with NR1-1a/NR2A, a significant proportion of the expressed NR1 subunits exists in an intracellular pool of unassembled subunits (Chazot and Stephenson, 1997b). Thus, the apparent lack of effect on NR1 immunoreactivities may be a result of NR1 subunits being redistributed from the unassembled pool to the assembled receptor complexes with no change in the absolute level of total NR1 subunits. It has been suggested that the availability of the NR2 subunit is the rate-limiting factor in the expression of functional NMDA receptors (Bessho et al., 1994). Therefore, if the NR2 subunit is stabilised by PSD-95, then the pool of unassembled NR1 subunits may be accessed, resulting in an increase in receptor number. Alternatively, if the number of NR1 subunits in assembled receptors is small in proportion to the number of unassembled NR1 subunits, then even a modest increase in the level of assembled or cell surface NR1

would be masked by the level of total NR1 immunoreactivity.

Coexpression of PSD-95_{c-Myc} with NR1-1a/NR2 NMDA receptors results in a decreased affinity for L-glutamate as measured by L-glutamate enhancement of [³H]MK-801 radioligand binding

Coexpression of PSD-95_{c-Myc} with NR1-1a/NR2A yielded an increase in the EC₅₀ for the enhancement of [³H]MK-801 binding by L-glutamate compared with NR1-1a/NR2A without PSD-95_{c-Myc}. This increase appears sufficient to explain the apparent decreased affinity for the binding of [³H]MK-801 to NR1-1a/NR2A with PSD-95 because [³H]MK-801 displacement assays carried out in the presence of 1 mM instead of the standard 10 μ M L-glutamate resulted in K_i values that were not significantly different from that of NR1-1a/NR2A without PSD-95. These experiments suggest that PSD-95 induces a decreased sensitivity of NR1-1a/NR2A receptors to L-glutamate. They are in agreement with the functional studies of Yamada et al. (1999), who showed that NR1/NR2B receptors expressed in *Xenopus* oocytes showed a decreased sensitivity to L-glutamate. The EC₅₀ values were 1.39 and 5.76 μ M without and with PSD-95, respectively. Mutational studies showed that the decrease in L-glutamate sensitivity was dependent on an interaction between the C terminus of the NR2B subunit and the second PSD-95/Dlg/ZO-1 domain of PSD-95 (Yamada et al., 1999). It was speculated that PSD-95 may play a protective effect in neuronal excitotoxicity (Yamada et al., 1999).

The EC₅₀ values determined here for NR1-1a/NR2A with and without PSD-95 for the stimulation of [³H]MK-801 binding by L-glutamate, 1.8 and 8.9 μ M without and with PSD-95, respectively, are in good agreement with the values determined by Yamada et al. (1999) for the L-glutamate sensitivities of NR1/NR2B channels with and without PSD-95. They differ, however, ~10- and ~40-fold from the EC₅₀ without and with PSD-95, respectively, for the L-glutamate stimulation of [³H]MK-801 binding found by Ransom and Stec (1988) for rat cerebral cortical membranes, i.e., EC₅₀ = 0.218 μ M. The discrepancy in EC₅₀ values may be explained by differences between native and cloned receptor preparations. PSD-95, as described above, is not the only NMDA receptor-associated protein.

The mechanism by which PSD-95 induces this change in L-glutamate sensitivity is not yet clear. It is possible that following expression in HEK 293 cells, PSD-95 associates with an endogenous protein such as a kinase or phosphatase, thus bringing the NMDA receptor and the modulatory enzyme into close proximity, resulting in regulation of NMDA receptor function. It is well established that both NR1 and NR2 subunits can undergo phosphorylation resulting in the modulation of NMDA receptor function (for review, see, e.g., Raymond, 1998). Indeed, coexpression of NR1/NR2B with PSD-95 in *Xenopus* oocytes resulted in the inhibition of protein kinase C-mediated potentiation of the NMDA receptor

channels. Furthermore, PSD-95 has been shown to link Fyn tyrosine kinase to NR2A subunits, resulting in enhanced levels of phosphorylated NR2A subunits (Tezuka et al., 1999). PSD-95 also associates with other members of the Src family of protein tyrosine kinases, including Src, Yes, and Lyn (Tezuka et al., 1999), as well as the brain-specific tyrosine phosphatase PTP ζ (Kawachi et al., 1999). Alternatively, PSD-95 may alter the binding properties of NMDA receptors purely as a consequence of its interaction with the NR2 subunits and the formation of receptor clusters. PSD-95 (alternative nomenclature, SAP90) has been shown to cluster GluR6 and GluR6/KA2 glutamate receptors on coexpression in mammalian cells and to alter receptor function by reducing desensitisation (Garcia et al., 1998).

In conclusion, we have used biochemical approaches to demonstrate that PSD-95 both enhances the expression of NR2A and NR2B subunits and, importantly, decreases the L-glutamate sensitivity of NR1-1a/NR2A receptors. These results complement the functional studies of Yamada et al. (1999). They suggest a role for PSD-95 in the determination of the number and efficacy of NMDA receptors that are expressed at synapses. Furthermore, they demonstrate that caution should be exercised in the correlation of the properties of neurotransmitter receptors expressed in isolation in nonneuronal cells to the properties of native receptors localised at synapses.

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