

***MOLECULAR CHARACTERISATION OF THE N-METHYL-D-
ASPARTATE RECEPTOR EXPRESSED
IN MAMMALIAN CELLS***

by

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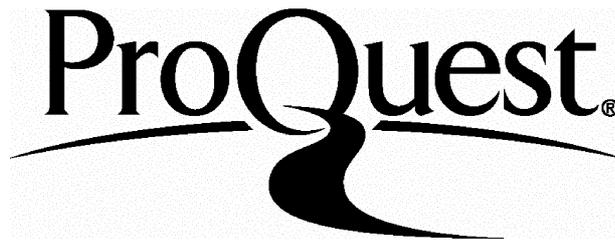
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ABSTRACT

L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system, mediating its effects via interaction with glutamate receptors. Several pharmacological subclasses of the glutamate receptor have been identified, including the N-methyl-D-aspartate (NMDA) receptor. Molecular cloning has recently identified five genes encoding the NMDA receptor subunits, NMDAR1 and NMDAR2A-D. In this thesis, work is described in which conditions for the optimal transient expression of homo- and heteromeric NMDA receptors in mammalian cells were established, thus providing a model system for structure-function studies of this important brain protein. Thus, the cDNAs encoding the NMDAR1-1a and NMDAR2A subunits were subcloned into the mammalian expression vector, pCIS. The resultant constructs were transfected alone or in combination in human embryonic kidney (HEK) 293 cells by the calcium phosphate method. Co-transfection studies resulted in cell death, which was prevented by inclusion of NMDA receptor antagonists in the cell culture medium post-transfection. A study was made of the efficacy for the prevention against cell death for a series of different NMDA receptor antagonists, which included DL-2-amino-5-phosphonovalerate (AP5), 5,7-dichlorokynurenic acid (DKA) and Mg^{2+} . The percentage cell death was quantified by either Trypan Blue exclusion or the CytoTox 96TM assay system. The combination of AP5 and DKA gave optimal protection with no significant difference between co-transfected and control samples. Site-directed *in vitro* mutagenesis was used to generate point mutations of the NMDAR1-1a subunit. Co-transfection studies with the wild-type and mutant NMDA receptor cDNAs were carried out and the effect of the mutations on cell viability quantified. Most notably, it was found that the mutation (N598Q), previously shown to reduce the Ca^{2+} permeability of cloned receptors, significantly reduced cell toxicity, thus providing evidence for the role of Ca^{2+} in NMDA-receptor mediated cytotoxic mechanisms.

*To Mum,
with love and happy memories*

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ABBREVIATIONS

1 _S ,3 _R -ACPD	1-Aminocyclopentane-1 _S ,3 _R -dicarboxylic acid
AHP	After-hyperpolarization
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AP3	2-Amino-3-phosphonopropionic acid
AP4	2-Amino-4-phosphonobutanoate
AP5	D-2-Amino-5-phosphonopentanoate
B _{max}	Maximum receptor density
bp	Base pairs
cAMP	Adenosine 3',5'-monophosphate
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CIP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus
7CK	7-Chlorokynurenic acid
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central Nervous System
CPP	3-((\pm)-2-Carboxypiperazine-4-)propyl-1-phosphonic acid
DAP	α,ϵ -Diaminopimelate
dATP	Deoxy-adenosine-5'-triphosphate
dATP α S	Deoxy-adenosine-5'-[α S]thiotriphosphate
dCTP α S	Deoxy-cytidine-5'-[α S]thiotriphosphate
ddNTPs	Dideoxynucleoside 5'-triphosphates
DEAE	Diethylaminoethyl
DKA	5,7-Dichlorokynurenic acid
DME/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham
DMSO	Dimethyl sulphoxide
<u>E. coli</u>	<u>Escherichia coli</u>
EDAC	1-Ethyl-3-(-dimethylaminopropyl) carbodiimide
EtBr	Ethidium bromide

FCS	Foetal calf serum
GABA	γ -Aminobutyric acid
GBP	Glutamate binding protein
G-protein	Guanine nucleotide-binding regulatory protein
HA-966	3-Amino-1-hydroxy-pyrrolidone-2
HBSS	Hanks' balanced salt solution
HEK	Human embryonic kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IP ₃	1,4,5-Trisphosphate
IPTG	Isopropyl- β -D-thiogalactopyranoside
I/V	Current-voltage
KBP	Kainate binding protein
K _D	Dissociation constant
L-680,560	(\pm)-Trans-2-carboxy-5,7-dichlorotetrahydroquinoline-4-phenylurea
L-AP4	l-2-Amino-4-phosphonobutanoic acid
LDH	Lactate dehydrogenase
LMP	Low melting point
LTP	Long term potentiation
MCPG	(+)- α -Methyl-4-carboxyphenylglycine
NBQX	2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline
NMDA	N-Methyl-D-aspartate
OD	Optical density
PCP	Phencyclidine
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RSV	Rous sarcoma virus
S.D.	Standard deviation
SDS	Sodium dodecyl sulphate

SKF-10,047	N-Allylnormetazocine
ssDNA	Single-stranded DNA
TCP	1-[1-(2-Thienyl)-cyclohexyl] piperidine
T _m	Melting temperature
TM	Transmembrane
TPA	12- <i>O</i> -Tetradecanoylphorbol 13 acetate
x-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

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Single channel recordings were done by Dr. Peter Stern, University College London.

Preliminary results with heteromeric NMDAR1-1a/NMDAR2C and NMDAR1-1a/NMDAR2A/NMDAR2C receptors were obtained by Sarah Coleman.

CHAPTER 1

General Introduction

1.1 FOREWORD

The brain, the most complex structure known, is the final frontier of biology. The human brain contains at least 10^{12} cells, $\sim 10^{11}$ of them neurons. An average neuron forms about 1 000 synaptic connections and receives even more. There, therefore, are about 10^{14} synaptic connections which are formed in the brain, a number which can be compared with the number of stars in our galaxy. Although almost a century ago, the existence of chemical messengers was postulated by John Langley and Henry Dale on the basis of their pharmacological studies (reviewed in Kandel et al., 1991), it is interesting that the first experiment which proved that a chemical transmitter was responsible for signal transmission in the synapse between excitable cells was designed from a dream of the scientist, Otto Loewi, in 1920. In his own words,

" ...The next night, at three o'clock, the idea returned. It was the design of an experiment to determine whether or not the hypothesis of chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory and performed a simple experiment on a frog heart according to the nocturnal design.....These results unequivocally proved that the nerves do not influence the heart directly but liberate from their terminals specific chemical substances which, in their turn, cause the well-known modifications of the function of the heart characteristic of the stimulation of its nerves. " (from Kandel et al., 1991).

Loewi called this substance *Vagusstoff* (vagus substance), which was soon after identified chemically as acetylcholine. Since then other chemical transmitters, called neurotransmitters, have been described in the Central Nervous System (CNS). In the CNS, γ -aminobutyric acid (GABA) and glycine are the major inhibitory neurotransmitters whereas glutamate (L-glutamate), is the major excitatory neurotransmitter. Upon neuronal stimulation of the presynaptic cell, the neurotransmitter is released from the presynaptic terminal into the synaptic

cleft (Figure 1.1). The main mode of neurotransmitter release in the synapse cleft of the CNS is by a Ca^{2+} -dependent mechanism. Ca^{2+} entry into the presynaptic terminal leads to a fusion of neurotransmitter-containing vesicles with the terminal membrane and release of neurotransmitter, but the mechanism by which Ca^{2+} exerts its effect is not known (Llinas *et al.*, 1992). Upon release into the synaptic cleft, the neurotransmitter interacts specifically with receptor proteins changing their conformation. This conformation change leads to an alteration in ion flux across the cell membrane or it activates the secondary messenger system. After acting on the neurotransmitter receptor, the neurotransmitter is removed from the synaptic cleft by two mechanisms. The most common one is by an uptake carrier into nerve terminals and glial cells, e.g. for dopamine, serotonin, GABA, glutamate or glycine or it can be degraded by an extracellular enzyme, such as acetylcholinesterase, which converts the neurotransmitter acetylcholine into choline and acetate. It has been also proposed that an uptake carrier can run backwards and in certain conditions serve as an alternative, Ca^{2+} -independent mechanism for neurotransmitter release (e.g. Adam-Vizi, 1992).

1.2 GLUTAMATE AS A NEUROTRANSMITTER

In the late 1950s, Curtis and his colleagues demonstrated that the amino acids L-glutamate and L-aspartate evoked repetitive discharges from all types of interneurons located in the dorsal horn and intermediate nucleus of the spinal cord of the cat (Curtis *et al.*, 1959). Since then it has been shown that glutamate fulfils the criteria for a neurotransmitter (e.g. Fonnum, 1984). Glutamate is the most abundant amino acid in the adult CNS but it does not cross the blood brain barrier. Instead, glutamate is synthesized by different biochemical routes within the brain. Some of these routes are: synthesis in the Krebs cycle from α -ketoglutarate by transamination, from α -ketoglutarate by the enzyme glutamic acid dehydrogenase and from glutamine by glutaminase (Siegel *et al.*, 1989). For

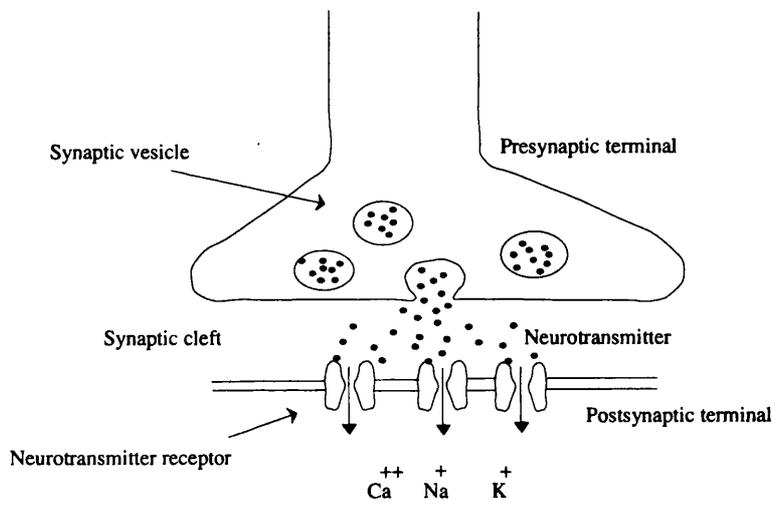


Figure 1.1 **Basic elements of chemical synaptic transmission**

the majority of brain regions, the most probable source seems to be formation from glutamine, which is produced from glutamate taken up into glial cells (Schousboe *et al.*, 1993). The uptake carrier transports two sodium ions with each glutamate anion into neurons and glial cells, in exchange for one potassium ion and one hydroxide ion (Bouvier *et al.*, 1992; Nicholls, 1993).

1.3 MOLECULAR PHARMACOLOGY OF GLUTAMATE RECEPTORS

Since evidence was first provided that glutamate is an excitatory neurotransmitter, many important actions of it have been revealed. In the vertebrate CNS, glutamate mediates fast synaptic transmission at the majority of excitatory synapses (Fonnum, 1984). Glutamate has also been shown to have an important role in early development of the nervous system (Mattson *et al.*, 1988), synaptic plasticity and memory formation (Bliss and Collingridge, 1993), seizures (Dingledine *et al.*, 1990) and neuronal degeneration (Meldrum and Garthwaite, 1990). On the basis of pharmacological and electrophysiological analyses, vertebrate glutamate receptors have been divided into two major groups termed ionotropic and metabotropic glutamate receptors. The ionotropic receptors were subdivided into the N-methyl-D-aspartate (NMDA)-selective subtype, the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-selective subtype, and the kainate-selective subtype. These three subtypes contain glutamate-gated, cation-specific ion channels. AMPA and kainate receptors are often referred to as non-NMDA receptors. The metabotropic receptors are the guanine nucleotide-binding regulatory protein (G-protein)-linked receptors associated with phosphoinositol metabolism and regulation of adenylyl cyclase activity. The fifth glutamate receptor subtype is the L-AP4 subtype, which has been proposed to explain the effects of l-2-amino-4-phosphonobutanoic acid (L-AP4) on inhibition of the synaptic transmission (Schoepp and Johnson, 1989).

1.3.1 NMDA RECEPTOR

The NMDA receptor ion channel complex was defined initially by the action of the selective agonist, NMDA, which was found to be 1000 fold more potent than glutamate in evoking excitatory responses (reviewed in Watkins and Olverman, 1987). NMDA binding opens an integral ion channel which is permeable to Ca^{2+} as well as to Na^+ and K^+ . Activation of NMDA receptors by agonist requires the presence of the co-agonist, glycine. The agonist binding site is blocked by the competitive antagonists D-2-amino-5-phosphonopentanoate (AP5) and 3-((±)-2-carboxypiperazine-4-)propyl-1-phosphonic acid (CPP). Several distinct modulatory sites of action for non-competitive antagonists have been identified on the NMDA receptor complex (Figure 1.2). The Mg^{2+} block site gives the NMDA receptor channel complex a unique property, blockade in a voltage-dependent manner by physiological concentrations of Mg^{2+} . Dissociative anaesthetics, such as phencyclidine (PCP, known on the streets as angel dust), ketamine and MK801 have been shown to block NMDA receptors (Anis *et al.*, 1983; Wong *et al.*, 1986). An inhibitory binding site of another divalent cation, Zn^{2+} , was reported but in contrast to Mg^{2+} , this inhibition was relatively voltage-independent (Reynolds and Miller, 1988). Polyamine, neurosteroid and ethanol modulatory sites have also been described (Ransom and Stec, 1988; Wu *et al.*, 1991; Gonzales and Hoffman, 1991).

1.3.1.1 The glycine binding site

It was firstly reported by Johnson and Ascher, (1987) that glycine potentiates the NMDA receptor responses of mouse central neurons in primary culture at a concentration as low as 10 nM. This potentiation was seen as an increase in the frequency of NMDA receptor channel opening. The potentiation produced by glycine was not antagonised by strychnine, thus demonstrating that

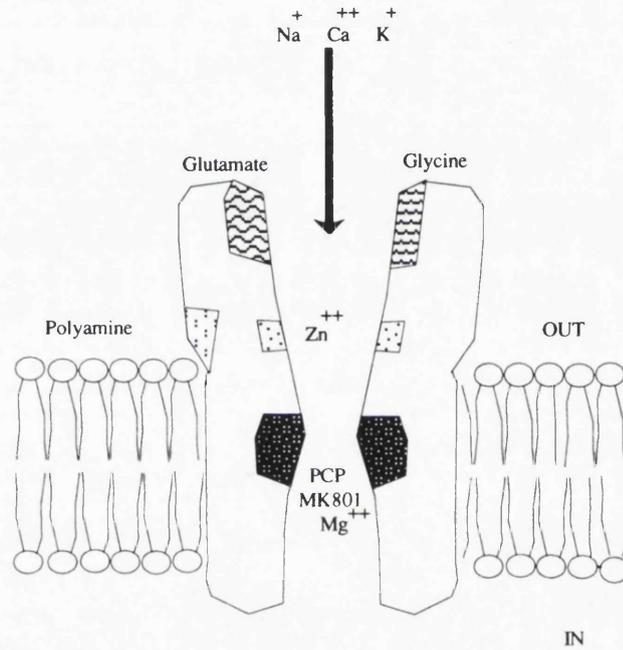


Figure 1.2

Schematic representation of the drug binding sites of the NMDA receptor-channel complex
(After Nakanishi, 1992)

the action of glycine does not involve the strychnine-sensitive receptor that mediates the classical inhibitory glycine response (Hamill *et al.*, 1983). Glycine binding to the NMDA receptor was shown to be absolutely required for NMDA activation of ionotropic glutamate receptors in *Xenopus* oocytes injected with rat brain mRNA (Kleckner and Dingledine, 1988). Ligand binding studies have demonstrated that glycine increases affinity of [³H]MK801 and [³H]TCP binding to the phencyclidine site of the NMDA receptor in a strychnine-insensitive manner (Wong *et al.*, 1987; Snell *et al.*, 1987). Regional distribution of strychnine-insensitive [³H]glycine binding site showed that this site is closely associated with the NMDA receptor complex (McDonald *et al.*, 1990). It was revealed at that time that the existing antagonist of the NMDA receptor 3-amino-1-hydroxy-pyrrolidone-2 (HA-966) was in fact selective for the glycine site, but it is now known that HA-966 is a partial agonist of the glycine site, with low efficacy (Foster and Kemp, 1989). Soon, more potent antagonists were developed with many of them being derivatives of kynurenic acid. Substitutions of a chlorine in the 5,7 positions resulted in one of them, 5,7-dichlorokynurenic acid, (DKA), (McNamara *et al.*, 1990). It was demonstrated that the DKA is a potent antagonist of the glycine site on the NMDA receptor, and that it reduced NMDA-mediated neurotoxicity in a cell culture model (McNamara *et al.*, 1990). The most potent antagonist to date is (±)-trans-2-carboxy-5,7-dichlorotetrahydroquinoline-4-phenylurea (L-680,560), (Grimwood *et al.*, 1992). [³H]L-680,560 was shown to bind to rat brain membranes with a K_D of 2.97 nM.

1.3.1.2 The divalent cation binding sites

The unique property of the NMDA receptor is the voltage-dependent Mg^{2+} channel block. The first demonstration that Mg^{2+} was a channel-blocker of the NMDA receptor complex came in the late 1970s (Evans *et al.*, 1978). Further analysis of this effect by two independent groups showed that Mg^{2+} action was

voltage-sensitive and that gradual relief of Mg^{2+} block occurred with membrane depolarization (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The K_D for Mg^{2+} at a membrane potential of 0 mV is 0.99 mM measured in whole-cell experiments (Mayer *et al.*, 1989) and 8.8 mM measured in single-channel recording (Ascher and Nowak, 1988). A second Mg^{2+} binding site was also proposed (Johnson and Ascher, 1990). It was shown that intracellular Mg^{2+} ions blocked outward current flow through NMDA receptors and that this effect became stronger with depolarization. The K_D for Mg^{2+} for this site, at membrane potential of 0 mV is 8.0 mM (Johnson and Ascher, 1990). It was also shown that extracellular and intracellular Mg^{2+} could block simultaneously with different kinetics. At the normal resting potential, -80 mV, the NMDA receptor channel is blocked by Mg^{2+} . For channel opening, besides binding of the agonist and co-agonist, membrane depolarization (to +30 mV) is required. When open, the channel has a high conductance of 50 pS and it is permeable to Ca^{2+} as well as to Na^+ and K^+ . Due to the requirement of membrane depolarization for NMDA receptor channel opening, the excitatory postsynaptic potential (EPSP) largely depends on activation of AMPA-kainate receptors, with only a small late component due to activation of NMDA receptors.

Another important property of the NMDA receptor is that the NMDA receptor component of the EPSP decays over hundreds of msec whereas the fast AMPA-kainate receptor component lasts a few msec at most. Glutamate remains bound to the NMDA receptor for an increased time compared to the AMPA-kainate receptor, due to its respective higher affinity and slower dissociation rate (reviewed in Jahr and Lester, 1992). The NMDA receptor channel opens repeatedly during this time and result is clusters of openings seen in steady-state single channel recordings (Gibb and Colquhoun, 1991). Rebinding of glutamate does not occur during the EPSP because no antagonism was observed when AP5 was applied within a few msec after presynaptic release of neurotransmitter (Lester *et al.*, 1990).

A second divalent cation site on the NMDA receptor has been described (Westbrook and Mayer, 1987). Zn^{2+} decreases channel activation and its action is partially voltage-dependent, which suggested a different site of action from Mg^{2+} . This site preferentially binds Zn^{2+} , but also recognizes Cd^{2+} and Hg^{2+} (Westbrook and Mayer, 1987).

Radioligand binding studies have also shown interactions between divalent cations and the NMDA receptor complex. It was demonstrated that Zn^{2+} and Cd^{2+} inhibit binding of [3H]glutamate to the NMDA receptor in contrast to Ca^{2+} and Mg^{2+} which do not inhibit [3H]glutamate binding (Monahan and Michel, 1987). The existence of the different binding site for Zn^{2+} was also suggested by Reynolds and Miller, (1988). They showed that the potency of Mg^{2+} increased in the presence of glutamate and glycine, while the potency of Zn^{2+} decreased.

1.3.1.3 The polyamine binding site

A polyamine binding site was firstly described by Ransom and Stec, (1988). They showed that spermine and spermidine in the cellular concentration range dramatically increased [3H]MK801 binding. This polyamine modulation was independent of the glutamate and glycine binding sites. They also showed that spermidine did not displace [3H]glycine and [3H]CPP binding to the NMDA receptor complex thus suggesting that polyamines are positive allosteric modulators of the NMDA receptor. It was shown that spermine increased the affinity of [3H]MK801, with no change in the number of binding sites (Williams *et al.*, 1989). Further characterization of the modulatory effects of polyamines has led to the classification of these compounds as agonists, partial agonists, antagonists and inverse agonists at the polyamine recognition site (Williams *et al.*, 1989; Reynolds and Miller, 1989; Williams *et al.*, 1990). Differential sensitivity of the NMDA receptor to polyamines during postnatal development has been reported (Williams *et al.*, 1991). Polyamines have also an effect on the

function of the NMDA receptor. For example, spermine increased the response elicited by NMDA in cultured hippocampal slices and in Xenopus oocytes injected with rat brain mRNA (Araneda et al., 1993; McGurk et al., 1990).

1.3.1.4 The PCP binding site

In the early 1980s, it was shown that dissociative anaesthetics, ketamine and PCP, selectively reduced excitation of central mammalian neurons by NMDA (Anis et al., 1983). Later, it was suggested that ketamine and PCP may block the ion channel operated by activation of the NMDA receptor (Honey et al., 1985). With the demonstration that the anticonvulsant, MK801, is a potent NMDA receptor antagonist, it was suggested that the MK801 binding site may also be associated with the channel within the NMDA receptor-channel complex (Wong et al., 1986). Therefore, it was proposed that MK801, ketamine and PCP acted at the separate non-competitive antagonist site PCP site within the NMDA receptor channel (Figure 1.2; Kemp et al., 1987). The two most selective ligands of the PCP binding site are [³H] 1-[1-(2-thienyl)-cyclohexyl] piperidine ([³H]TCP) and [³H]MK801 (Vignon et al., 1986; Wong et al., 1986). MK801 was identified using a screen for anticonvulsant activity and it is one of the most potent antagonists with *in vitro* affinity in the 1-10 nM range. MK801 is also very specific for the NMDA receptor. In radioligand binding studies [³H]MK801 has a high ratio of total to nonspecific binding making it the ligand of choice in assaying the NMDA receptor. Because PCP binding site exists within the ion channel, only when glutamate and glycine are bound to the NMDA receptor complex is the ion channel in the appropriate configuration for the PCP ligands TCP and MK801 to bind (Ransom and Stec, 1988).

1.3.2 NON-NMDA RECEPTORS

With the development of agonists and antagonists for glutamate receptors, it became evident from the late 1960s onwards that there may be more than a single type of glutamate receptor. With the isolation of kainic acid from the marine alga, Digenia simplex, it emerged that NMDA and kainate activated different types of glutamate receptors (reviewed in Watkins and Olverman, 1987). Soon after the discovery of kainate, another agonist, quisqualate was isolated from seeds of the plant Quisqualis. Major advances occurred in the late 1970s when a selection of new NMDA antagonists was described, including Mg^{2+} , HA-966 and α,ϵ -diaminopimelate (DAP). Use of these pharmacological tools yielded a new classification of NMDA, quisqualate and kainate subtypes of the glutamate receptor. However, when an agonist with a greater selectivity for quisqualate receptor was described, namely AMPA, this receptor was renamed as the AMPA receptor (Krogsgaard-Larsen et al., 1980). As the NMDA receptor was the first described, AMPA and kainate receptors are often referred to as non-NMDA receptors. No specific antagonists for kainate and AMPA receptors have been reported, although two of them, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) show some selectivity (Honoré et al., 1988; Sheardown et al., 1990). CNQX showed a five- and NBQX a thirty-fold higher affinity respectively, for the [3H]AMPA binding sites compared to the [3H]kainate binding site.

Receptor autoradiographic studies using [3H]AMPA and [3H]kainate have revealed a differential distribution of these two sites (reviewed in Young and Fagg, 1990). [3H]AMPA binding distribution corresponded closely to the distribution of NMDA receptors suggesting that these two receptors together activate the postsynaptic neuron. Some of the brain regions with high levels of [3H]AMPA binding sites include the hippocampus, the cerebral cortex, and the molecular layer of the cerebellum (Olsen et al., 1987). [3H]Kainate binds to high

and low affinity sites within the vertebrate CNS. Distribution of the high affinity kainate binding site was different to those of AMPA or NMDA sites but it corresponded well to the brain regions vulnerable to the neurotoxic action of kainate such as cerebral cortex, the hippocampal CA3 area and the lateral septum (Unnerstall and Wamsley, 1983). As data describing AMPA and kainate receptors were accumulating, some authors suggested the existence of a unitary non-NMDA receptor in lower vertebrates (Barnard and Henley, 1990). Still there was also strong evidence for the existence of separate kainate receptors on mammalian spinal cord C fibres, insensitive to quisqualate and AMPA (Evans *et al.*, 1987) and high affinity kainate binding sites in mammalian brain (K_D 2-50 nM), poorly inhibited by AMPA (Krogsgaard-Larsen *et al.*, 1980).

Low micromolar concentrations of kainate are required to elicit an excitatory response. At those concentrations, kainate also interacts with AMPA receptors, thus it was suggested that the neurophysiological effects of kainate may be mediated via the AMPA receptor (Young and Fagg, 1990). Indeed, it was shown that kainate activates AMPA receptors with a slow desensitization time-course (Patneau and Mayer, 1991). Kainate also activates high affinity kainate receptors with a fast desensitization time-course (Huettner, 1990). Both AMPA and kainate gate low-conductance cation channels (less than 20 pS), that are permeable to Na^+ and K^+ (Cull-Candy and Usowicz, 1987). Electrophysiological experiments showed that fast synaptic transmission was often blocked by non-NMDA antagonists suggesting that AMPA-kainate receptors may be the depolarizing receptors at many excitatory synapses (Mayer and Westbrook, 1987). Moreover, it has been shown that currents induced by activation of AMPA-kainate receptors were potentiated by the activation of adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase A (PKA), (Wang *et al.*, 1991; Greengard *et al.*, 1991).

Unitary kainate-AMPA, and kainate-AMPA-NMDA receptors were purified from the Xenopus CNS (Henley *et al.*, 1992), supporting evidence for

a unitary receptor at least in lower vertebrates. Indeed, molecular cloning has revealed the existence of 11 non-NMDA receptor subunits that can assemble in homomeric and heteromeric configurations to form two different receptors, the AMPA receptor and the high-affinity kainate receptor (reviewed in Seeburg, 1993). Therefore, molecular cloning has yielded a molecular basis for the observed pharmacological heterogeneity reported for the mammalian brain.

1.3.3 METABOTROPIC GLUTAMATE RECEPTOR

The involvement of glutamate in the stimulation of phosphoinositide metabolism in CNS was discovered in the 1980s (Sladeczek *et al.*, 1985). It was shown that glutamate and quisqualate potently stimulated the hydrolysis of membrane phosphoinositides by activating phospholipase C thus producing the second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, IP₃. This action of glutamate was mediated by G-protein linked metabotropic receptors (reviewed in Baskys, 1992). It has also been demonstrated that metabotropic glutamate receptors can be negatively coupled to adenylyl cyclase (Prezeau *et al.*, 1992). Several nonspecific agonists bind to metabotropic receptors, including quisqualate, ibotenate, glutamate, with the specific agonist being 1-aminocyclopentane-1s,3R-dicarboxylic acid (1s,3R-ACPD or trans-ACPD). No high affinity specific antagonists are available for metabotropic receptors at the present time. The compounds, 2-amino-3-phosphonopropionic acid (AP3) and 2-amino-4-phosphonobutanoate (AP4) are partial antagonists but only in the mM concentration range (Hwang *et al.*, 1990). Recently, a new antagonist of metabotropic glutamate receptors negatively coupled to adenylyl cyclase has been reported, (+)- α -methyl-4-carboxyphenylglycine (MCPG) (Kemp *et al.*, 1994). The effect of metabotropic glutamate receptor agonists has been described in several parts of the brain and also in *Xenopus* oocytes expressing rat brain mRNA (Sugiyama *et al.*, 1987). It has been demonstrated that Purkinje cells possess very

high concentration of metabotropic glutamate and IP₃ receptors (Hwang *et al.*, 1990) also, cultured astrocytes from many regions of brain have been demonstrated to possess these receptors (Cornell-Bell *et al.*, 1991). Electrophysiological studies have revealed that metabotropic glutamate receptors are involved in blockade of a slow after-hyperpolarization (AHP) in CA1 hippocampal neurons. Action potentials in CA1 neurons are followed by a slow AHP mediated by Ca²⁺-activated K⁺ conductance, thus limiting the ability of the cell to fire action potentials. This effect is known as spike accommodation (reviewed in Miller, 1991). It has also been proposed that metabotropic glutamate receptors have a role in two processes, long-term potentiation (LTP) in the hippocampus and long-term depression in the cerebellum (Anwyl, 1991).

Molecular cloning has revealed the existence of at least six subtypes of metabotropic glutamate receptors, mGluR1-6 (e.g. Nakanishi 1992). It has been reported that the mGluR1 and mGluR5 stimulate IP₃ formation and intracellular Ca²⁺ mobilisation. The subtypes mGluR2-4 and mGluR6 were reported to inhibit the forskolin-stimulated accumulation of intracellular adenosine 3',5'-monophosphate (cAMP) in agonist-dependent manner (Nakanishi 1992).

1.4 MOLECULAR BIOLOGY OF NON-NMDA RECEPTORS

1.4.1 CLONING OF NON-NMDA RECEPTORS

Molecular cloning has identified 9 genes encoding subunits of non-NMDA glutamate receptors, GluR1-7 and KA1-2. The landmark of molecular cloning was the isolation of the complementary DNA (cDNA) encoding the GluR1 subunit by Heinemann and his colleagues in 1989 (Hollmann *et al.*, 1989). The GluR1 cDNA was isolated from a rat forebrain cDNA library by functional expression in *Xenopus* oocytes. The GluR1 cDNA has an open reading frame of 2.7 kb and the deduced amino acid sequence was found to have similarities with

the chick and frog kainate binding protein (KBP) cDNAs which were described at the same time (Wada *et al.*, 1989; Gregor *et al.*, 1989). The GluR1 subunit consists of 889 amino acids with a calculated M_r of 99 769 which was the largest molecular mass for a ligand-gated ion channel subunit reported at that time. Protein sequence comparisons with other members of the ligand-gated ion channel superfamily, such as nicotinic acetylcholine receptor revealed little homology (Betz, 1990). Hydropathy analysis of the GluR1 amino acid sequence predicted four hydrophobic segments of sufficient length to form transmembrane α -helices TM 1-4 (Figure 1.3) Shortly after the GluR1 cDNA was cloned, 3 other cDNAs encoding putative glutamate receptors were isolated from a rat brain cDNA library (Keinänen *et al.*, 1990). They were named GluR2-GluR4 (Figure 1.3). The predicted polypeptides were each approximately 900 amino acids in length with 70 % overall amino acid sequence identity. A splice variant of the GluR4 subunit with an additional C-terminal segment of 38 amino acids designated GluR4c, has also been described (Gallo *et al.*, 1992). Other laboratories have also reported cloning of the mouse AMPA subunits (Sakimura *et al.*, 1990), and the rat AMPA subunits (Nakanishi *et al.*, 1990). It was interesting to note that for all GluR subunits the region between TM3 and TM4 was extremely well conserved (Figure 1.3), in contrast to the corresponding region of other members of ligand-gated ion channel superfamily, which show high sequence diversity within this region. The first subunit of the kainate receptor subclass to be cloned was the GluR5 cDNA (Bettler *et al.*, 1990). Amino acid sequence comparison with the GluR1-4 subunits showed only 40 % sequence identity. Other members of this kainate subclass were subsequently cloned, GluR6 (Egebjerg *et al.*, 1991) and GluR7 (Bettler *et al.*, 1992). GluR5-7 subunits share ~ 70 % sequence identity. Two members of the high affinity kainate receptors have also been cloned, the KA1 cDNA (Werner *et al.*, 1991) and the KA2 cDNA (Herb *et al.*, 1992, Sakimura *et al.*, 1992). Sequence comparison showed only ~ 40 % sequence identity with the GluR5-7 subclass.

**Figure 1.3 Comparison of polypeptide sequences encoded by the rat
AMPA receptor cDNAs (GluR1-GluR4)**

Identical amino acids are represented by (*), conservative substitutions are represented by (.). The putative transmembrane regions (TM1-4) are shown by (■). The flip/flop segment is shown by (—). The leading peptide sequence and the Q/R site are indicated. The sequence alignment was performed by the PC/GENE software package using the method developed by Higgins and Sharp (1988).

leading peptide → 1		
MPYIFA----FFCTGFLGAVVG	ANFPNNIQIGGLFPNQSQEHAAFRALSQLTE	33
M-QKIMHISVLLSPVLWGLIFG	V-SSNSIQIGGLFPRGADQEYSAFRVGMVQFST	32
MGQSVLRAVFFLVGLLGHSHG	G-FPNTISIGGLFMRNTVQEHSFAFRFAVQLYNT	32
M-RIICRQIVLLFSGFWGLAMG	A-FPSSVQIGGLFIRNTDQEYTAFLAIFLHNT	32
* *	* *	
PP-----KLLPQIDIVNISDTFEMTYRFC	SQFSKGVYAI FGFYERRTVNMLTS	81
SE-----FRLTPHIDNLEVANSFAVTNAFCSQ	FSRQVYAI FGFYDKKSVNTITS	81
NQNTTEKPFHLNYHVDHLDSSNSFSVTNAFCSQ	FSRQVYAI FGFYDQMSMNTLTS	87
SPNASEAPFNLVPHVDNIETANSFAVTNAFCSQ	YSRQVFAIFGLYDKRSVHTLTS	87
* *	* *	
FCGALHVCFITPSFPVDTSNQFVLQLRPELQEAL	SIIIDHYKWQTFVYIYDADRG	136
FCGTLHVSFITPSFPDGTGHPFVIQMRPDLK	GALLSLIEYYQWDFAYLYDSDRG	136
FCGALHTSFVTPSPFPTDADVQFVIQMRPALK	GAILSLLSYKWEKFFVLYDTERG	142
FCSALHISLITPSFPTEGESQFVLQLRPSLRG	ALLSLDHYEWNCFVFLYDTERG	142
* *	* *	
LSVLQRVLDTAAEKNWQVTAVNILTTEE----	GYRMLFQDLEKKKERLVVVDCE	187
SLSTLQAVLDSAAEKWQVTAINVGNINNDK	KDETYRSLFQDLELKKERRVILDCE	191
FSVLQAIMAAVQNNWQVTARVGNIKD----	VQEFRRIEEMDRRQEKRYLIDCE	194
YSILQAIMKAGQNGWHVSAICVENFND----	V-SYRQLEELDRRQEKKFVIDCE	193
* *	* *	
SERLNAIQIVKLEKNGIGYHYILANLGFMDID	LNKFKESGRNVTGFQLVNYTD	242
RDKVNDIVDQVITIGKHVKGYHYIIANLGF	TDGDLKIQFGGANVSGFQIVDYDD	246
VERINTILEQVVILGKHSRGYHYMLANLGF	TDILLERVMHGGANITGFQIVNEN	249
IERNQILQIVSVGKHVKGYHYIIANLGF	KDISLERFIHGGANVTGFQLVDFNT	248
* *	* *	
TIPARIMQWRTSDSRDHRVDWKRPKYTSALT	YDGVKVMMAEAFQSLRRQRIDIS	297
SLVSKFIERWSTLEEKEYPGAHTATIKYTSAL	TYDAVQVMTAEFRNLRRQRIEIS	301
PMVQQFIQRWRLDEREFPEAKNAPLKYTSAL	THDAILVIAEAFRYLRRQRVDVS	304
PMVTKLMDRWKKLDQREYPGSETPP-KYTSAL	TYDGVLMMAETFRSLRRQKIDIS	302
* *	* *	
RRGNAGDCLANPAVPWQGQIDIQRALQQVRF	EGLTGNVQFNEKGRRTNYTLHVIE	352
RRGNAGDCLANPAVPWQGVIEIERALKQVQV	EGLSGNIKFQDQNGKRINYTINIME	356
RRGSAGDCLANPAVPWSQGIDIERALKMVQV	QGMTGNIQFDTYGRRTNYTIDVYE	359
RRGNAGDCLANPAAPWQGLDMERTLKQVRIQ	GLTGNVQFDHYGRRVNYTMDVFE	357
* *	* *	
MKHDGIRKIGYWNEDDKFVPAATDAQAGD	NSSVQNRITYVTTILEDPYVMLKKN	407
LKTNGPRKIGYWSEVDKMVVTLTELP	SGNDTSGLENKTVVVTILESPYVMMKKN	411
MKVSGSRKAGYWNEYERFVPSDQQ-ISND	SSSENRTIVVTTILESPYVMYKKN	413
LKSTGPRKVGWYNDMDKLVLIQDMPTL	GNDTAAIENRTVVVTTIMESPYVMYKKN	412
* *	* *	
ANQFEGNDRYEGYCVLAAEIAKHVGYSYR	LEIVSDGKYGARDPDTKAWNGMVGE	462
HEMLEGNERYEGYCVDLAAEIAKHCGFKY	KLTIVGDGKYGARDADTKIWNGMVGE	466
HEMLEGNERYEGYCVDLAYEIAKHVRIKY	KLIVGDGKYGARDPETKIWNGMVGE	468
HEMLEGNDKYEGYCVDLASEIAKHIGIKY	IAIVPDGKYGARDADTKIWNGMVGE	467
* *	* *	
LVYGRADVAVAPLTITLVREEVIDFSKPF	MSLGLSISIMIKKPQKSKPGVFSFLDPL	517
LVYGKADIAIAPLTITLVREEVIDFSKPF	MSLGLSISIMIKKPQKSKPGVFSFLDPL	521
LVYGRADIAVAPLTITLVREEVIDFSKPF	MSLGLSISIMIKKPQKSKPGVFSFLDPL	523
LVYGKAEIAIAPLTITLVREEVIDFSKPF	MSLGLSISIMIKKPQKSKPGVFSFLDPL	522
* *	* *	
TM1		
AYEIMWCIVFAYIGSVVFLVSRFSPYEW	HSEEFEEGRD--QTSDQSNEFGIF	570
AYEIMWCIVFAYIGSVVFLVSRFSPYEW	HTEEFEDGRETQ--SSESTNEFGIF	574
AYEIMWCIVFAYIGSVVFLVSRFSPYEW	HLEDNNEEPRDPQSPDPNEFGIF	578
AYEIMWCIVFAYIGSVVFLVSRFSPYEW	HTEEPEDGKEGP--SDQPPNEFGIF	575
* *	* *	

TM2 TM3

NSLWFLGAFMQQGCDISPRSLSGRIVGGVWFFTLIIISSYTANLAAFLTVERM 625
 NSLWFLGAFMRQGCDISPRSLSGRIVGGVWFFTLIIISSYTANLAAFLTVERM 629
 NSLWFLGAFMQQGCDISPRSLSGRIVGGVWFFTLIIISSYTANLAAFLTVERM 633
 NSLWFLGAFMQQGCDISPRSLSGRIVGGVWFFTLIIISSYTANLAAFLTVERM 630

↑
 Q/R site

VSPIESAEDLAKQTEIAYGTLDSGSTKEFFRRSKIIVFEKMWTYMKSAPSVFVL 680
 VSPIESAEDLSKQTEIAYGTLDSGSTKEFFRRSKIIVFDKMWTYMRSAEPSVFVR 684
 VSPIESAEDLAKQTEIAYGTLDSGSTKEFFRRSKIIVYEKMWSYMKSAPSVFTK 688
 VSPIESAEDLAKQTEIAYGTLDSGSTKEFFRRSKIIVYEKMWTYMRSAEPSVFTR 685

TTEEGMIRVRKTKGKYAYLLESTMNEYIEQRKPCDTMKVGGNLDKSGYGIATPKG 735
 TTAEGVARVRKSKGKYAYLLESTMNEYIEQRKPCDTMKVGGNLDKSGYGIATPKG 739
 TTADGVARVRKSKGKFAFLESTMNEYIEQRKPCDTMKVGGNLDKSGYGVATPKG 743
 TTAEGVARVRKSKGKFAFLESTMNEYTEQRKPCDTMKVGGNLDKSGYGVATPKG 740

"flip or flop" segment

SALRNPVNLAVLKLNEQGLLDKLNKWWYDKGECGTGGGDSKDKTSALSLSNVAG 790
 SSLGNVNLAVLKLNEQGLLDKLNKWWYDKGECGSGGGDSKEKTSALSLSNVAG 794
 SALGNVNLAVLKLNEQGLLDKLNKWWYDKGECGSGGGDSKDKTSALSLSNVAG 798
 SSLGNVNLAVLKLNEQGLLDKLNKWWYDKGECGSGGGDSKDKTSALSLSNVAG 795

TM4

VFYILIGGLGLAMLVALIEFCYKSRSESKRMKGFLIPQQSINEAIRTSTLPRNS 845
 VFYILVGGGLGLAMLVALIEFCYKSRAEAKRMK----- 826
 VFYILVGGGLGLAMMVALIEFCYKSRAESKRMK-----LT----- 832
 VFYILVGGGLGLAMLVALIEFCYKSRAEAKRMK-----LTFSEATRNC----- 837

GAGASGGGGSGENGRVVSQDFPKSMQSI PSMSSHSSGM-PLGATGL 889 GluR1_RAT
 -----VAKNPQINIPSSSQNSQNFATYKEGYNVYGIESVKI 862 GluR2_RAT
 -----KNTQNFKPAPATNTQNYATYREGYNVYGTESVKI 866 GluR3_RAT
 -ARLSITGVSGENGRVLTDPCKAVHTGTAIRQSSGLAVIASDLP 881 GluR4_RAT
 *

1.4.2 PROPERTIES OF EXPRESSED NON-NMDA RECEPTORS

Upon *in vitro* transcription of the GluR1 cDNA and injection of the resultant mRNA into Xenopus oocytes, the kainate-gated functional homomeric receptors were expressed (Hollmann *et al.*, 1989). The chick and frog KBP which were cloned at the same time as the GluR1 subunit, did not form functional ion channels upon expression in mammalian cells. Although, the KBP cloned from frog brain formed a high affinity kainate binding site, when expressed in mammalian cells (Wada *et al.*, 1989). To analyze the properties of GluR1-4 subunits, homomeric and heteromeric receptors were expressed transiently in human embryonic kidney (HEK) 293 cells and Xenopus oocytes. When radioligand binding studies were performed on transiently transfected cells, only [³H]AMPA specific binding was observed (Keinänen *et al.*, 1990). Results obtained with GluR2 subunit alone showed that high affinity [³H]AMPA binding site (K_D 12 nM) which was in good agreement with the high affinity [³H]AMPA site within the brain (Insel *et al.*, 1990). Although these subunits displayed characteristic AMPA radioligand pharmacology, their electrophysiological response to both AMPA and kainate supported the concept that many of the electrophysiological effects of kainate are mediated by AMPA receptors (Rassendren *et al.*, 1990). Electrophysiological analysis of the co-expressed GluR1 and GluR2 subunits, showed activation by glutamate, quisqualate, AMPA and kainate. Similar electrophysiological responses to agonists were later obtained with the GluR1-GluR3 subunits, singly expressed in Xenopus oocytes by the Heinemann group (Boutler *et al.*, 1990). They have also examined the current-voltage (*I/V*) relations for the kainate activated channels in oocytes expressing single subunit or combinations of subunits. When single GluR1 or GluR3 subunits or the combination of GluR1 and GluR3 subunits were expressed, the *I/V* curves showed strong inward rectification and reversal

potential near -40 mV, in contrast to the linear *I/V* curve and reversal potential of about -10 mV, obtained in oocytes injected with brain poly(A⁺) RNA. When GluR2 subunit was coexpressed with GluR1 or GluR3, or when all three subunits were coexpressed together, *I/V* curves were more similar to ones obtained with oocytes expressing the hippocampal poly(A⁺) RNA, with combination GluR1 and GluR2 showing nearly linear *I/V* curve and reversal potential of about -10 mV.

Further characterization of the functional properties of AMPA receptor subunits revealed some unexpected features. Firstly, a small segment preceding the predicted fourth transmembrane region was shown to exist in two versions with different amino acid sequences (Figure 1.3, Sommer *et al.*, 1990). This segment consists of 38 amino acids and is located within the highly conserved region among AMPA subunits. The two alternative versions were named "flip" and "flop". DNA sequencing revealed that the flip and flop sequences were on adjacent exons separated by an intron of approximately 900 bp, indicating that flip and flop versions of all four subunits were generated by alternative mRNA splicing. The electrophysiological characteristics of different splice variants, flip and flop, were analyzed upon co-expression of the GluR1 and GluR2 subunits in the mammalian cell line. The flip and flop variants differed in their responses to glutamate and AMPA. Glutamate activated channel four to five times more effectively when flip versions were expressed, but similar amplitude differences between the flip and flop variants were produced with glutamate and AMPA. Similar results were obtained when GluR1 and GluR3 subunits were co-expressed.

A second unexpected feature of AMPA receptor subunits was that the *I/V* properties of receptor channel described above and the Ca²⁺ permeability, depend on the single amino acid. The Ca²⁺ permeability of AMPA receptor subunits was studied in *Xenopus* oocytes (Hollmann *et al.*, 1991). It was showed that the homomeric GluR1, GluR3 channels or channels co-assembled with the GluR1 and GluR3 subunits were permeable to Ca²⁺. In contrast channels co-assembled

with the GluR2 subunit together with the GluR1 or GluR3 showed no Ca^{2+} permeability. The putative TM2 region is identical in each of the four GluR subunits, except that GluR2 carries a positively charged arginine residue (R) at amino acid position 586, whereas a glutamine residue (Q) is found in the homologous position in GluR1, GluR3 and GluR4 subunits (Q/R site, Figure 1.3). This single amino acid residue within TM2 was identified by site-directed mutagenesis to determine Ca^{2+} permeability (Mishina *et al.*, 1991). The same residue (R586) was identified to determine the steady-state (*I-V*) behaviour of the AMPA receptor channel described above (Verdoorn *et al.*, 1991). Further analysis of AMPA subunits gene sequences revealed the third unexpected feature (Sommer *et al.*, 1991). Although an arginine codon (CGG) was found in GluR2 mRNA at the position which determines channel ion-flow (R586), at the same position, the genomic DNA sequence is harbouring a glutamine codon (CAG). It was proposed that the codon change is generated by RNA editing. Efficiency of this process is remarkable since GluR2 subunit undergoes editing to an extent exceeding 99 %. A polymerase chain reaction (PCR) analysis has detected increase of unedited GluR2 cDNA at embryonic day 14, suggesting a developmental regulation of this process (Burnashev *et al.*, 1992a).

When expressed in HEK 293 cells, the homomeric GluR5 channels exhibited properties of high affinity domoate (K_D 2 nM) and kainate (K_D 70 nM) binding site (Sommer *et al.*, 1992). The channels were also gated in decreased order of sensitivity by domoate, kainate, glutamate and high concentration of AMPA. The first description of GluR5 cDNA cloning, reported existence of two presumably splicing forms differing in the presence (GluR5-1) or absence (GluR5-2) of a 45 bp insert located in the predicted N-terminal domain (Bettler *et al.*, 1990). Sommer has reported the existence of three additional splice variants (GluR5-2a, 2b and 2c) (Sommer *et al.*, 1992). These variants differed in sequences distal to the putative TM4. The pharmacological behaviour of these splice variants was the same but 5-10 fold higher B_{max} values were obtained with

the shortest C-terminal form (GluR5-2a). The RNA editing described to modulate GluR2 mRNA, was also observed for GluR5 and GluR6 subunits, but to lesser extent (Sommer et al., 1991). The GluR5 subunit was shown to contain TM2-specific arginine codon only in 40 % of cDNAs, and GluR6 in 80 %. In addition to RNA editing in TM2, GluR6 had two positions in TM1 that were modified by RNA editing (Köhler et al., 1993). These positions were isoleucine (edited to valine, I-V) and tyrosine (edited to cysteine, Y-C). Occurrence of these forms are variable in CNS. The GluR6 subunit fully edited in TM1 and TM2 positions amounts to 65 % of all GluR6 subunit in rat brain. The genomically encoded, unedited form constitutes only 10 %, with other forms constituting the remaining 25 % of GluR6 subunits. It was shown that only when the TM1 positions are edited, that the editing of TM2 (R590Q) influences Ca^{2+} permeability. But in contrast to AMPA subunits where the TM2 with arginine at the critical position are impermeable to Ca^{2+} , GluR6(R) channels edited in TM1 showed higher permeability than GluR6(Q) channels. When TM1 positions were unedited, changes in Ca^{2+} permeability, as a consequence of TM2 editing, were much less pronounced.

The subunits GluR7, KA1 and KA2 failed to generate functional homomeric channels. However, co-expression of KA2 subunit with both, GluR5 and GluR6 subunits generated agonist-sensitive channels (Herb et al., 1992, Sakimura et al., 1992). The combination of GluR5 and KA2 subunits transiently expressed in HEK 293 cells produced heteromeric channels that showed more rapid desensitization and different *I-V* relations when compared to homomeric GluR5 channels suggesting that these two subunits co-assemble. An interesting feature of the heteromeric GluR6 / KA2 receptor channels was their response to AMPA, which did not gate homomeric GluR6 channels. Homomericly expressed KA1 and KA2 subunits exhibited high affinity kainate binding sites with dissociation constants of 3.9 nM and ~ 15 nM respectively (Werner et al., 1991; Herb et al., 1992).

1.4.3 **DISTRIBUTION OF NON-NMDA RECEPTORS**

In situ hybridization revealed a differential pattern of expression of the mRNAs encoding the GluR1-4 subunits. For example, GluR2 was shown to be expressed in all layers of the cerebral cortex while GluR1, GluR3 and GluR4 expression clearly differed among layers. The flip and flop variants had also different expression patterns, as revealed by *in situ* hybridization using oligonucleotide probes specific for the alternatively spliced variants. These differences were particularly evident in the hippocampus. The CA3 neurons had only flip variant of GluR1-GluR3, while both splice variants of all four subunits exist in CA1 neurons. The dentate gyrus had a higher expression of flop version relative to flip. During brain development, differences in the expression of the flip and flop variants were also observed (Monyer et al., 1991). The flip forms of GluR1-GluR4 were mostly expressed in the prenatal brain whereas flop forms appeared only from early postnatal stages onwards. *In situ* hybridisation analyses of GluR5-7, KA1 and KA2 subunits revealed a complex mosaic that approximate to the autoradiographic pattern observed for high affinity kainate sites in the rat brain (reviewed in Seeburg, 1993).

1.4.4 **IMMUNOCHEMICAL CHARACTERIZATION OF THE NON-NMDA GLUTAMATE RECEPTORS USING SUBUNIT SPECIFIC ANTIBODIES**

Several groups have reported the development of subunit-specific antibodies against the non-NMDA receptor subunits, GluR1-GluR4, and their use in the identification of molecular composition of the GluR complex in rat brain (Wenthold et al., 1992; Molnár et al., 1993; Petralia and Wenthold, 1992; Blackstone et al., 1992; Martin et al., 1993). Results obtained with antibodies made to synthetic peptides corresponding to sequences at the C-termini of

GluR1-4 subunits suggested that native glutamate receptors in the rat brain exist as hetero-oligomers made up of GluR1-4 subunits (Wenthold *et al.*, 1992). Analysis of the purified receptor using GluR2-3-selective antibody showed a major immunoreactive band at M_r 108 000, with some additional higher molecular weight immunoreactive components. The largest component migrated at M_r 590 000 suggesting that native AMPA receptors have a pentameric structure similar to that of the other ligand-gated ion channels. Antibodies made to synthetic peptides corresponding to different regions of GluR1 subunit were also used to purify receptors from the rat brain membranes (Molnár *et al.*, 1993). These probes recognised a specific immunoreactive band at M_r 105 000 in immunopurified samples. By using sequence specific antibodies, both groups concluded that the C-terminus of the functional receptor molecule has an intracellular location, contrary to the currently held model (Petralia and Wenthold, 1992; Molnár *et al.*, 1993). Antipeptide antibodies that recognize the C-terminal domains of GluR1, GluR2-3-4c and GluR4, detected on immunoblots distinct proteins with M_r ranging from 102 000 to 108 000 in homogenates of rat brain (Martin *et al.*, 1993). Also a comprehensive study of GluR1-4 receptor immunocytochemistry was carried out on sections of rat brain. It showed that GluR1-4 subunits are distributed abundantly and differentially within neuronal cell bodies and processes in rat brain, with precise patterns and cellular localization unique for each subunit (Petralia and Wenthold, 1992; Molnár *et al.*, 1993; Martin *et al.*, 1993). The overall pattern of GluR1-4 subunits distribution correlated well to the previously described distribution of mRNAs encoding for GluR1-4 subunits (Pellegrini-Giampietro *et al.*, 1991) and to previous studies on the distribution of [3 H]AMPA binding in rat brain (Olsen *et al.*, 1987). The subunits were visualized only postsynaptically suggesting that AMPA receptors do not have a major role as presynaptic autoreceptors.

1.5 MOLECULAR BIOLOGY OF NMDA RECEPTORS

1.5.1 CLONING OF NMDA RECEPTORS

Because of the importance of NMDA receptors in brain physiology and pathophysiology, cloning of the NMDA receptor was a long awaited event. Because solubilisation and purification of NMDA receptors proved to be very difficult and partial protein sequence was not obtained (e.g. Ikin *et al.*, 1990), alternative approaches were used to clone the NMDA receptor. Using a cloning technique combined with functional expression in *Xenopus* oocytes, Nakanishi's group isolated a single cDNA clone (λ N60) from a rat forebrain cDNA library (Moriyoshi *et al.*, 1991). This clone encoded the NMDAR1 subunit of the NMDA receptor complex. Soon after, other subunits of the NMDA receptor complex were isolated, NMDAR2A-2D (e.g. Meguro *et al.*, 1992; Monyer *et al.*, 1992). The nucleotide sequence of the NMDAR1 showed a large open-reading frame of 2.8 kb encoding a polypeptide of 938 amino acids with a calculated relative molecular mass of 105 000. The sequence comparison with the GluR1 subunit showed overall homology of about 22-26 %. On the basis of hydrophobicity analysis, four transmembrane regions were predicted with amino and carboxy ends proposed to be extracellular, although later experimental evidence suggested an intracellular location for the carboxy terminal (Figure 1.4, see later, 1.5.2). Together with the cloning of the mouse counterpart of the NMDAR1 subunit, named NMDA ζ 1, the presence of a smaller form of the NMDAR1 subunit was suggested (Yamazaki *et al.*, 1992). The smaller subunit had deletion of 37 amino acids (deletion II, residues 846-882, Figure 1.5) and it was designated as NMDAR1-2a (Hollmann *et al.*, 1993). Several laboratories have subsequently described the existence of 8 isoforms of the NMDAR1 subunit generated by alternative splicing, with the first cloned NMDAR1 subunit named NMDAR1-1a (summarized in Figure 1.5, Sugihara *et al.*, 1992; Anantharam *et*

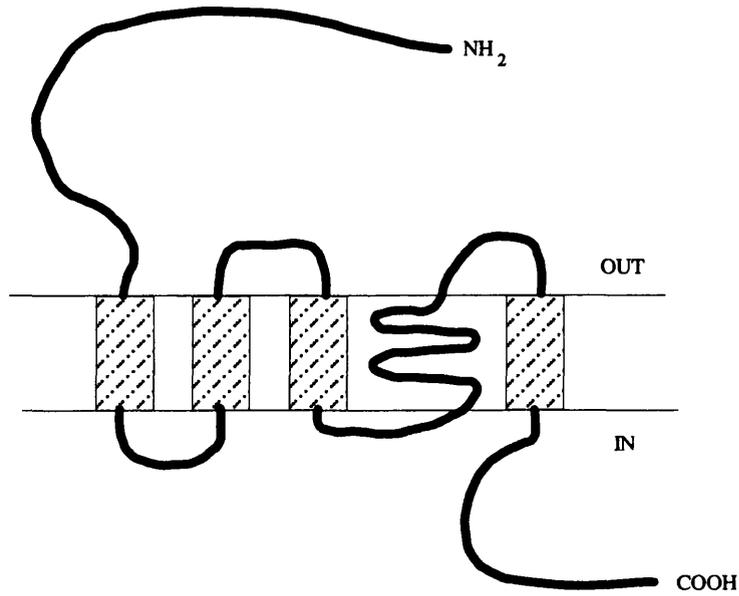


Figure 1.4

Schematic diagram of the predicted transmembrane topology of a glutamate ionotropic receptor subunit (After Seeburg, 1993)

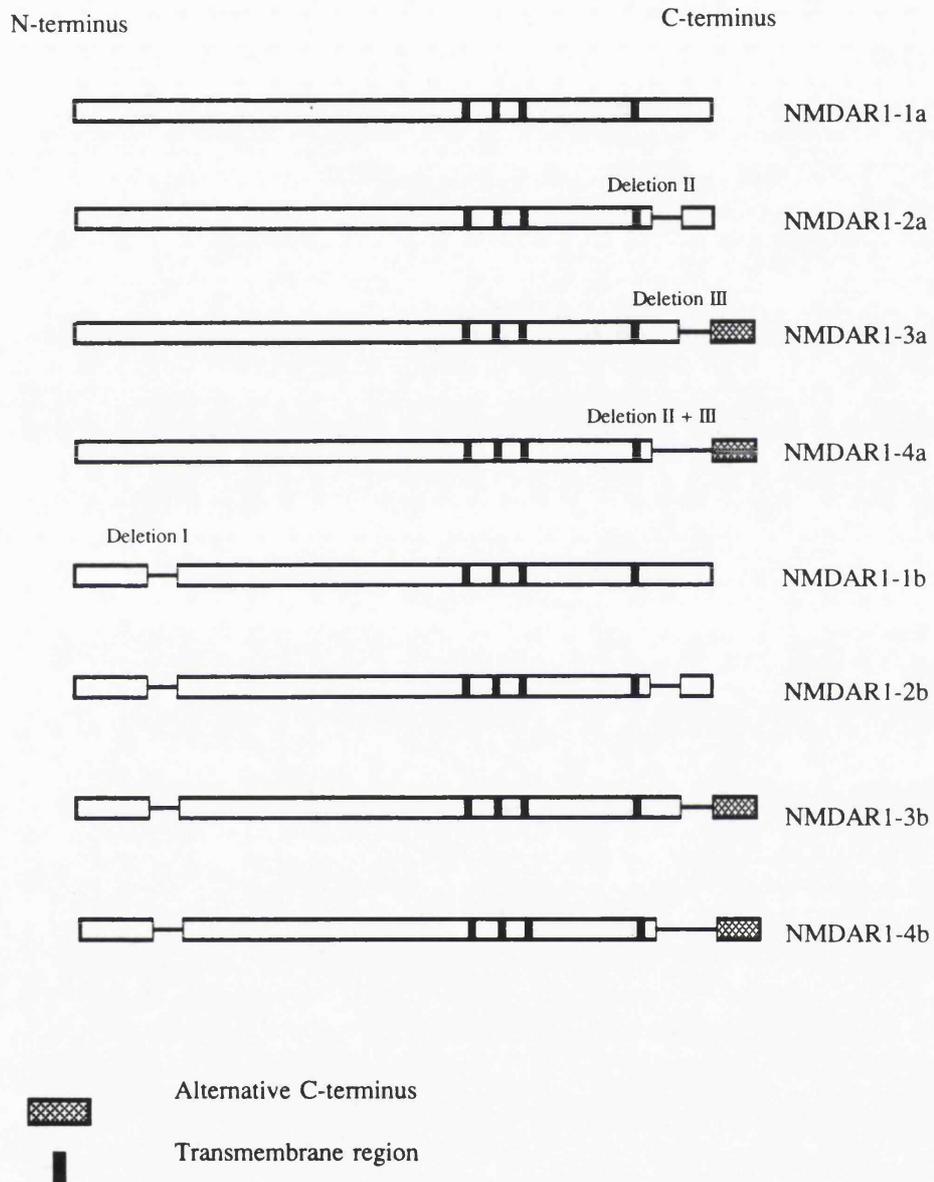


Figure 1.5 Schematic representation of the NMDAR1 splice variants.
(After Hollmann *et al.*, 1993)

al., 1992; Durand *et al.*, 1992; Durand *et al.*, 1993; Nakanishi *et al.*, 1992; Hollmann *et al.*, 1993). All of these isoforms had the same amino acid sequence with differences in the insertion or deletion of 3 additional exons. Only the isoforms with deletion III, generated a different C-terminus (isoforms R1-3a, R1-3b, R1-4a and R1-4b).

The first member of a new subunit type, NMDAR2A (named $\epsilon 1$) was isolated by screening of a mouse forebrain cDNA library using mouse AMPA-kainate cDNAs as probes (Meguro *et al.*, 1992). Following that cloning, 3 other members of the NMDAR2 subtype were isolated from rat and mouse brain libraries using a screening technique and PCR amplification (R2B, R2C and R2D, Figure 1.6, Monyer *et al.*, 1992; Kutsuwada *et al.*, 1992; Meguro *et al.*, 1992; Ikeda *et al.*, 1992 and Ishii *et al.*, 1993). The NMDAR2A-R2D subunits share ~ 50 % amino acid sequence identity and they have only ~ 15 % amino acid sequence identity to the NMDAR1 subunit. The new subunits had a very long C-terminus with more than 600 amino acids for R2A and R2B subunits. The proposed mature R2A-R2D subunits were composed of 1 445, 1 456, 1 220 and 1 296 amino acids with calculated molecular masses of 163 267, 162 875, 133 513 and 140 656 respectively. The hydrophobicity analysis suggested four putative transmembrane segments, TM1-TM4 located in the middle of the molecule (Figure 1.6). As in the NMDAR1 subunit, the important position for Ca^{2+} permeability, in the TM2 region was occupied by an asparagine.

By using a different approach, Kumar and his colleagues described cloning of the cDNA for one of putative subunits of the NMDA receptor complex, the glutamate binding protein (GBP, Kumar *et al.*, 1991). Previously, they have reported partial purification of GBPs that formed ligand-gated ion channels when reconstituted into liposomes (Ly and Michaelis, 1991). With a modification of that procedure, they have purified a complex of proteins with all the ligand binding characteristics of the NMDA receptor complex. Using polyclonal and monoclonal antibodies raised against 71 K GBP, they have screened a rat

Figure 1.6 Comparison of the polypeptide sequences encoded by the mouse NMDAR1, R2A and R2C receptor cDNAs

Identical amino acids are represented by (*), conservative substitutions are represented by (.). The putative transmembrane regions (TM1-4) are shown by (■). The leading peptide sequence and the N598 site are indicated. The sequence alignment was performed by the PC/GENE software package using the method developed by Higgins and Sharp (1988).

	leading peptide →	1	
NMDAR1	MSTM---HLLTFALLFSCSFA	RAAC-----DPKIVNIGAVLSTRKHEQMFREAV	28
NMDAR2A	MG-RLGYWTLVLVLPALL-VWHGPA	QNAAAEEKGTPALNIAVLLGHS--HDVTEREL	29
NMDAR2C	MGGALG--PALLLTSLLGAWA	G----LGAGQGEQAVTVAVVFGSS--GPLQAQAR	28
	* . . . *	
R1	NQANKRHGSKWIKLNATSVT---HKPNAIQMALSVCEDLISSQVYAILVSHPPPTP		80
R2A	RNLWGPEQATGLPLDVNVVALLMNRTDPKSLITHVCDLMSGARIHGLVFGD--DT		82
R2C	TRL-TPQNFLDLPLEIQPLTIGVNNTNPSSILTQICGLLGAARVHGIVFED--NV		80
	
R1	NDHFTPTPVSYTAGFYRIPVLGLTTRMS-IYSDKSIHLSFLRTVPPYSHQSSVWF		134
R2A	DQEAVAQMLDFISSQTFIPILGIHGGASMIMADKDPSTSTFFQFGASIQQATVML		137
R2C	DTEAVAQLLDFVSSQTHVPILSISGGSAVVLTPKEPGSAFLQLGVSLEQQQLQVLF		135
	
R1	EMRVYNWNHIILLVSDDEGRAAQKRLETLLLEER--ESKAEKVLQFDPG---TK		184
R2A	KIMQDYDWHVFSLVTTIFPGYRDFISFIKTTVDNSFVGWDMQNVTILDTSFEDAK		192
R2C	KVLEEYDWSAFVITSLHPGHALFLEGVRAVADASYLSWRLLDVLTLLELPGGGPR		190
	
R1	NVTALLMEARDLEARVILLSAEDDAATVYRAAAMLNMTGSGYVWLVGEREISGN		239
R2A	TQVQ--L--KKIHSSVILLYCSKDEAVLILSEARSLGLTGYDFFWIVPSLVSGNT		243
R2C	ARTQRL--RQVDAPVLVAYCSREEAEVLFABAAQAGLVGPHVWLVPNLALGST		243
	
R1	ALRYA--PDGIIGLQLIN-GKNESAHISDAVGVAQAVHELLEK-ENITDPPRCG		290
R2A	ELIPKEFPSSGLISVSYDDWDYSLEARVRDGLILTTAASSMLEKFSYIPEAKASC		298
R2C	DAPPAAFPVGLISVVTESWRLSLRQKVRDGVAILALGAHSYRRQYGTLPAPAGDC		298
	
R1	VGNTN-IWKTGPLFKRVLMSKSYADGVTGRVEFNEDGDRKFANYSIMNLQNRKL-		343
R2A	YGQTEKPEPLHLTHQFMVNVTDGK---DLSFTEEGYQVHPRLVVIIVLNKDREW		350
R2C	RSHPGPVSPAREAFYRHLNVTWEGR---DFSFPGGYLVQPTMVVIALNRHRLW		350
	
R1	VQVGIYNGTHVIPNDRKIIWPGGETEKPRGYQMSTRLKIVTIHQEPFVYVK-PTM		397
R2A	EKVGKWE--NQTLRLRHAVWPYKSFSDCEPD-DNHLISIVTLEEAPFVIVEDIDP		402
R2C	EMVGRWD--HGVLYMKYPVWPRYSTSLQPVD-SRHLTVATLEERPFVIVESPDP		402
	
R1	SDGTCKEEFTVNGDPVKKVICTGPNDTSPGSPRHTVPQCCYGFCDVLLIKLARTM		452
R2A	LTETC---VRNTVPCRKFV--KIN-NSTNEGMNVKCKCGFCIDILKLSRTV		449
R2C	GTGGC---VPNTVPCRRQS---NHTFSSGDITPYTKLCKCGFCIDILKLAQV		450
	
R1	NFTYEVHLVADGKFGTQERVNNSNKKKEWNGMGEGLLSGQADMIVAPLTINNERAQ		507
R2A	KFTYDLYLVNTGKHGK--KVNNV---WNGMIGEVVYQRAVMAVGSILTINEERSE		498
R2C	KFSYDLYLVNTGKHGK--RVRGV---WNGMIGEVVYKRAMAIGSLTINEERSE		499
	
		TM1	
R1	YIEFSKPFKYQGLTILVKKEIPRSTLDSFMQPFQSTLWLLV-GLSVHVAVMLYL		561
R2A	VVDFSVPFVETGISVMVSRNGTVSPSAFLPEFSASVWMMFMVLLIVSAIAVAVFV		553
R2C	IIDFSVPFVETGISVMVARSNGTVSPSAFLPEYSPAVWMMFMCLTVVAITVFM		554
	
		TM2	
R1	LDRFSP--FGRFKVNSEEEEEEDALTLSSAMWFSWGLLNSGIGEGAPRSFSARIL		614
R2A	FEYFSPVGYNRNLAAGKAPHGSPFTIGKAIWLLWGLVFNNSVPVQNPKGTTSKIM		608
R2C	FEYFSPVSYNQNLTKGKSGGSPFTIGKSVLLWALVFNNSVPIENPRGTTSKIM		609
	
		↑	
		N(598) in NMDAR1	
	TM3		
R1	GMVWAGFAMIVASYTANLAFLVLDREPERITGINDPRLRNPSDK---FIYATV		666
R2A	VSVWAFVAVIFLASYTANLAAFMIQEEFVDQVTGLSDKKFQRPHDYSPFRFGTV		663
R2C	VLVWAFVAVIFLASYTANLAAFMIQEQYIDTVSGLSDKKFQRPDQYPPFRFGTV		664
	

R1 KQSSVDIYFRRQVELSTMYRHMEKHNYESAAEAIQAVRDNKLHAFIWD SAVLEFE 721
R2A PNGSTERNIRN--NYPYMHQYMTKFNQRGVEDALVSLKTGKLDAFIYDAAVLNYK 716
R2C PNGSTERNIRS--NYRDMHMHMVKFNQRSVEDALVSLKMGKLDAFIYDAAVLNYM 717
. * . . . * * * * * * * *

R1 ASQK--CDLVTTGE--LFFRSGFGIGMRKDSPWKQNVSL SILKSHENGFMEDLDK 772
R2A AGRDEGCKLVITIGSGYIFATGTGYGIALQKGS PWKRQIDLALLQFVGDGEMEELET 771
R2C AGKDEGCKLVITIGSGKVFATGTGYGIAMQKDSHWKRAIDLALLQFLGDGETQKLET 772
* . . . * . . . * . . . * . . . * . . . * . . . * . . . * . . . *

TM4

R1 TWVRYQECDSRSNA--PATLTFENMAGV FMLVAGGIVAGIFLIFI----- 815
R2A LWLT-GICHNEKNEVMSSQLDIDNMAGVFYMLAAAMALSLITFIWEHLFYWKLRF 825
R2C VWLS-GICHNEKNEVMSSKLDIDNMAGVFYMLLVAMGLALLVFAWEHLVYWKLRF 826
* . . . * . . . * . . . * . . . * . . . * . . . * . . . *

R1 -----EIA YKR-----HKDARRKQMQQLAFAAVN----- 838
R2A CFTGVCSDRPGLLFSISRGYISCIHG VHI---EKKKSPDFNLTGSQSNMLKLLR 877
R2C SVPS--SSQLDFLLAFSRGIYSCFNGVQSLPSPARPPSPDLTAGSAQANVLKMLQ 879
. . . * . . . * . . . * . . . * . . . *

R1 ----- 838
R2A SAKNISNMSNMSSRMDSPKRAADFIQRGSLI-VDMVSDKGNLIYSDNRSFQGKD 931
R2C AARDMVSTADVSGS-LDRATRITIE--NWGNRRAPAPTTSGRSCTPGPPGQSP 931

R1 -VWRKNLQDRK-----SGRAEPDP 856
R2A SIFGENMNELQTFVANRHKDSL SNYVFGQHPLTLNESNPNTVEVAVSTESKNS 986
R2C SGWRPPGGRTPLARRAPQPPARPGPAQGRLSPTCPEHPAGTLGMR-GGQCESGI 985
. *

R1 KKKATFRAITSTLA-----SSFKR----- 875
R2A RPRQLWKKSMESLRQDSL NQN-----PVSQRDEKTAENRTHSLKSPRYLP--- 1031
R2C RDRTSRPPERRALPERSLLHAHCHYSSFPRAERSGRPFLPLFPEPPEPDDLPLL 1040
. . . *

R1 -----RRSSKDTSTGGGRGALQNKDVTLPRAIE----- 905
R2A -EEVAHSDISETSSRATCHREPDNKNHKT KDNFKRSMASKYPKDCSEVERTYVK 1085
R2C PEQLARREALLRAAWARGPRPRHASLPSSVAEAFTRS--NPLPARCTGHACAC-- 1091
. * *

R1 -----REE 908
R2A TKASSPRDKIYTI DGEKEPSFHLDP PQFIENIVLPENVDFPDTYQDHNFNFRKGD 1140
R2C ---PCPQSRPSCRHVAQTQSLRL--PSYREACVEGVPAGVAATWQPRQHVC LHTH 1141
.

R1 GQLQLCS-----RHRES----- 920
R2A STLPMNRNPLHNEDGLPNNDQYKLYAKHFTLKD KGSPPH-- 1200 SEGSDRY 1185
R2C THLPFCWGTVCRHPPPCSSHSPWLIGTWEP PSHRGRTLGL 1200 GTGYRDSGVL 1191
. *

R2A RQNSTHCRSCLSNLPTYSGHFTMRS PFKCDA CLRMGNLYDIDEDQMLQETGNPAT 1240
R2C EEVSREACGTQGFPR SCTWRRISSLESEV 1220

R2A REEAYQDWSQNNALQFQKNL KINRQHSYDNILDKPREIDLSRPSRSISLKDRE 1295
R2A RLLEGNLYGSLFVSPSSKLLGNKSSLFPQGLEDSKRKSLLPDHTSDNPF LHTYG 1350
R2A DDQRLVIGRCPSPDPYKHS LPSQAVNDSYLRSSLRSTASYCSRDSRSHSDVYISEH 1405
R2A VMPYAANKNNMYSTPRVLN SCSNRRVYKMP SIESDV 1442

hippocampal cDNA expression library. One clone was selected which they suggest encoded for a subunit of the brain NMDA receptor.

The cloning of the putative presynaptic glutamate receptor subunit was also recently reported (Smirnova *et al.*, 1993). The clone, named GR33 encoding 288 amino acids was obtained from the rat cDNA library. The protein sequence was identical to that of the presynaptic protein syntaxin, which was suggested to dock synaptic vesicles near Ca^{2+} channels at presynaptic active zone (Bennett *et al.*, 1992). When expressed in *Xenopus* oocytes, GR33 formed apparent glutamate-activated ion channels with pharmacological properties similar to those of native NMDA receptors. The identity of this protein as a glutamate receptor is, however, highly controversial.

1.5.2 PROPERTIES OF EXPRESSED NMDA RECEPTORS

Cloning of the NMDAR1 subunit revealed that the NMDA receptor could be a homo-oligomer with all the pharmacological properties of the native NMDA receptor. However, co-expression of the NMDAR1 and one of the NMDAR2 subunits resulted in much larger electrophysiological responses following receptor activation by glutamate or NMDA. Also, hetero-oligomeric receptors showed different pharmacological profiles. Further diversity in pharmacological properties of cloned NMDA receptors arose from expression of different splice-variants of the NMDAR1 subunit. In contrast to NMDAR1 subunit, the NMDAR2A-D subunits expressed alone could not form a functional receptor.

When mRNA derived from the NMDAR1 cDNA was injected into *Xenopus* oocytes, NMDA (100 μM) elicited an inward current of 40-70 nA (Hollmann *et al.*, 1989). This current was almost completely abolished with 100 μM Mg^{2+} at holding potentials between -80 mV and -30 mV. This block was removed at holding potentials more positive than -20 mV, demonstrating the unique property of the NMDA receptor complex, the voltage-dependent Mg^{2+}

block. Glycine requirement was also demonstrated, but omission of glycine only partially reduced the NMDA response (Hollmann *et al.*, 1989). Different NMDA antagonists were tested for the inhibition of NMDA elicited response. Action of both competitive antagonists (e.g. AP5) and noncompetitive antagonists (MK801 and 7-Chloro-kynurenic acid) on the NMDA receptor was demonstrated (Hollmann *et al.*, 1989). In addition to the properties of the rat NMDAR1 described above, Yamazaki showed potentiation of glutamate response by polyamines and by 12-*O*-tetradecanoylphorbol 13 acetate (TPA) - activator of protein kinase C (PKC) (Yamazaki *et al.*, 1992). Potentiation by TPA was an important feature as it was suggested that PKC has an important role in induction and maintenance of LTP (Ben-Ari *et al.*, 1992).

The NMDAR1 isoforms were expressed in *Xenopus* oocytes and their properties were investigated (e.g. Hollmann *et al.*, 1993). Various antagonists of the NMDA receptor (AP5, CPP, Mg²⁺, MK801 and 7-chlorokynureate), effectively inhibited the NMDA-induced channel activity of the 8 isoforms without significant difference. It has been reported that NMDAR1 isoforms differ in affinity for NMDA and potentiation by spermine and activators of protein kinase C (Durand *et al.*, 1992; Durand *et al.*, 1993; Hollmann *et al.*, 1993). They showed that variants lacking the N-terminus insert, R1-1a, R1-2a, R1-3a, R1-4a, so-called "a" splice-variants, exhibited higher affinity for NMDA (K_D 12 μ M) and potentiation by spermine at high glycine concentration (10 μ M). These properties were in contrast to isoforms R1-1b, R1-2b, R1-3b and R1-4b, so-called "b" splice-variants, which had lower affinity for NMDA (K_D 42-67 μ M) and no spermine potentiation. At low glycine concentration (1 μ M) all isoforms could be potentiated by spermine. The isoforms also differed in the extent to which they could be potentiated by an activator of PKC (phorbol 12-myristate 13-acetate, PMA). The highest potentiation (20-fold) was shown by the isoform with the N-terminal insert and without the both C-terminal inserts (NMDAR1-4b), (Durand *et al.*, 1993). A difference between "a" and "b" splice variants was

also reported in their potentiation by submicromolar concentration of Zn^{2+} . As the inhibitory action of Zn^{2+} on the NMDA receptors was known, it was a surprise that the homomeric "a" splice variants expressed in oocytes were potentiated by 0.1-1 μM Zn^{2+} . However, heteromeric R1/R2A receptors in any splice variant combination and type "b" homomeric splice variants were not potentiated by Zn^{2+} at any concentration (Hollmann *et al.*, 1993).

It was also demonstrated that phosphorylation of the NMDAR1 receptor transiently expressed in HEK 293 cells, was regulated by alternative splicing of the receptor C-terminus (Tingley *et al.*, 1993). Four serine residues were identified within the first C-terminal insert as major phosphorylation sites for a PKC. When these residues were changed to an alanine residues, PKC phosphorylation of the mutants was greatly reduced. These results provided the first evidence that the C-terminus of the NMDAR1 subunit was located intracellularly, suggesting that the transmembrane topology model, based on hydropathy plots of the amino acid sequence, may be incorrect (Figure 1.4).

Single subunit expression of any of the R2A-R2D subunits in Xenopus oocytes, did not give detectable responses to glutamate or NMDA agonists in the presence of glycine. Co-expression of the NMDAR1 subunit with any of the NMDAR2 subunits elicited a large response to glutamate and NMDA, which on average was 10 - 100 fold larger than the response in oocytes expressing the NMDAR1 subunit alone. The exception was the NMDAR2D subunit which when co-expressed with the NMDAR1 subunit gave only a 5-fold increase in current amplitude (Ikeda *et al.*, 1992), and as reported by another group, no increase at all, probably due to translation problems (Ishii *et al.*, 1993). Co-expression of the NMDAR1 and NMDAR2A-D subunits also produced receptors with a variability in their affinity for agonists, the effectiveness of antagonists and the sensitivity to Mg^{2+} block (Monyer *et al.*, 1992; Kutsuwada *et al.*, 1992 and Ikeda *et al.*, 1992). The R1/R2D combination had the highest affinity for glutamate and glycine, with the order of the affinities being, R1/R2D > R1/R2C

> R1/R2B > R1/R2A. The sensitivity for the competitive antagonist of the glutamate binding site AP5 was also different. The most sensitive to AP5 was the R1/R2A combination, with the order of affinities being, R1/R2A > R1/R2B > R1/R2C > R1/R2D. The most sensitive combination to glycine site antagonist 7CK was R1/R2C combination. There was also a remarkable difference in sensitivity to Mg^{2+} block. The R1/R2C combination was resistant to Mg^{2+} block, being active even at -70 mV membrane potential in the presence of 1 mM Mg^{2+} . It was also shown that the heteromeric R1/R2A and R1/R2B, but not the R1/R2C and R1/R2D channels, were potentiated by treatment with TPA, a PKC activator (Kutsuwada *et al.*, 1992; Mori *et al.*, 1993). The construction of chimeric subunits between the NMDAR2B and NMDAR2C, has revealed that carboxyl-terminal region of the NMDAR2B subunit was responsible for the channel activation by TPA (Mori *et al.*, 1993). The sensitivity of heteromeric R1/R2 channels was also examined for non-competitive antagonists, such as MK801, PCP, ketamine and N-allylnormetazocine (SKF-10,047) (Yamakura *et al.*, 1993). It was shown that the heteromeric R1/R2A and R1/R2B receptor channels were more sensitive to MK801 than the R1/R2C and R1/R2D channels. The sensitivities to antagonists PCP, ketamine and SFK-10,047 were only slightly variable among the four R1/R2 combinations.

The single-channel properties of the NMDAR1 and NMDAR2A-C subunits co-expressed in *Xenopus* oocytes were characterized (Stern *et al.*, 1992). The single channel recordings of the R1/R2A and R1/R2B combinations showed very comparable properties to each other and these properties were similar to the NMDA receptor channels of hippocampal CA1, dentate gyrus and cerebellar granule cells. They had 50 pS opening conductances with 40 pS sublevels, similar lifetime and frequencies. The only difference observed was that the R1/R2A channels had lower glycine sensitivity than the native receptors. The single channel characteristics of the R1/R2C heteromeric channels were markedly different, with 36 pS openings and 19 pS sublevels, similar to native channels

of large cerebellar neurons in culture.

The NMDAR1 and NMDAR2A-D subunits clearly demonstrated the properties of a NMDA receptor complex. The evidence is still required to confirm the role of GBP and syntaxin as glutamate receptors.

1.5.3 DISTRIBUTION OF NMDA RECEPTORS

In situ hybridization has revealed that the NMDAR1 mRNA was widely expressed throughout the rat brain, in almost all neuronal cells (Ishii et al., 1993). It was also shown that the NMDAR2A-D subunits had differential expression patterns. They were more restricted in their expression pattern in comparison to the ubiquitous expression of the NMDAR1 subunit. The most widely expressed was the NMDAR2A subunit, with mRNA detected in many brain regions, such as: the cerebral cortex, the hippocampus, the caudate-putamen, the thalami and amygdaloid nuclei and granule cell layer of the cerebellar cortex. The NMDAR2B subunit was expressed only in the forebrain, with no mRNA detected in cerebellum. In contrast, NMDAR2C subunit had high level of expression in the granule cell layer of the cerebellum, with weak mRNA signal detected in olfactory bulb and thalamus. Only during period, postnatal day 7 to postnatal day 14, transient expression of the R2C was detected in the hippocampus (Pollard et al., 1993). Expression of the R2D subunit was restricted to the diencephalon and lower brain stem regions (Monyer et al., 1992; Kutsuwada et al., 1992; Watanabe et al., 1992; Meguro et al., 1992 and Ishii et al., 1993).

Anti-NMDAR1 polyclonal antibodies were used for immunocytochemical studies of the rat brain. Immunocytochemical localization of the NMDAR1 subunit demonstrated it to be prominent in all brain regions which agreed with results from *in situ* hybridization studies described above. Distribution of the NMDAR1 subunit was the most prominent in layers 2,3 and 5, of the cerebral

cortex, and in the hippocampus and cerebellum (Brose *et al.*, 1993).

1.5.4 IDENTIFICATION OF THE FUNCTIONAL DETERMINANTS OF THE NMDA RECEPTOR

It has been revealed by site-directed mutagenesis that amino acid arginine at position 586 in the putative TM2 region of the GluR2 subunit determines Ca^{2+} permeability of the AMPA receptors (Burnashev *et al.*, 1992a). The corresponding positions in the putative TM2 regions of the NMDAR1 and NMDAR2A-R2D subunits were occupied by an asparagine. To determine the importance of this residue for Ca^{2+} permeability and channel blockade, substitution mutations were introduced into the R1 and the R2 subunits by site-directed mutagenesis. When this position in R1 subunit was replaced by glutamine (N598Q) or arginine (N598R) heteromeric R1(N598Q)/R2B receptors were much less sensitive to Mg^{2+} block the corresponding mutation (N to Q), in R2B or in R2C had a similar effect (Mori *et al.*, 1992; Burnashev *et al.*, 1992b). When mutation (N to Q) was introduced in TM2 of R2A subunit, heteromeric R1/R2A(N595Q) had even more marked reduction in Mg^{2+} block than R1(N598Q)/R2A. When heteromeric R1(N598R) /R2A receptors were expressed, no Mg^{2+} blockade was observed even under highly hyperpolarized conditions (Sakurada *et al.*, 1993). Sensitivity to channel blocker MK801 was also changed with the described mutations. Mutations R1(N598Q) and R2B(N589Q) markedly reduced MK801 block and mutation R1(N598R) completely abolished MK801 blockade of the NMDA response. The mutations R1(N598Q), R1(N598R), R2B(N589Q) and R2B(N589R) had an effect on non-competitive antagonists PCP, ketamine and SKF-10,047, with N to Q mutation reducing the sensitivities of R1/R2A channels to all 3 antagonists, though to various extents. The N to R mutation abolished sensitivities of R1/R2A channels to all of these 3 antagonists (Yamakura *et al.*, 1993). The effect of tricyclic

antidepressant, desipramine, was affected by R1(N598Q) and R1(N598R) mutations in similar way to the non-competitive antagonists (Sakurada *et al.*, 1993). These two mutations only reduced sensitivity to 10 μM Zn^{2+} , but the mutant channels were strongly inhibited by 100 μM Zn^{2+} (Mori *et al.*, 1992; Sakurada *et al.*, 1993).

All of these mutant analysis suggested that non-competitive antagonists (PCP, ketamine, SKF-10,047) as well as MK801, Mg^{2+} and desipramine act on common site, within the NMDA receptor channel.

The string of five acidic amino acids at the N-terminal side of the TM2 in R1 subunit was replaced by corresponding amino acids of the GluR1 subunit without having any effect on Mg^{2+} , Zn^{2+} or MK801 inhibition (Mori *et al.*, 1992). When each of these five acidic amino acids was replaced by its amide derivative individually, none of these mutations had effects on Mg^{2+} and MK801 block or NMDA response either (Sakurada *et al.*, 1993).

1.6 GLUTAMATE IN PHYSIOLOGY AND PATHOPHYSIOLOGY

1.6.1 LTP AND MEMORY

LTP is an associative increase in synaptic strength as a result of high-frequency train of action potentials produced synchronously in a small population of neurons. In addition to the previously established role of glutamate in synaptic transmission, it was established in the early 1980s that NMDA glutamate receptors were necessary for the induction of LTP, which underlies at least certain forms of memory (Collingridge *et al.*, 1983; reviewed in Bliss and Collingridge, 1993). The first detailed description of LTP in the dentate area of the hippocampus, was published in the 1970s (Bliss and Lømo, 1973; Bliss and Garden-Medwin, 1973). Since then LTP has been found in all excitatory

pathways in the hippocampus (Bashir and Collingridge, 1992), as well as in the amygdala (Clugnet and LeDoux, 1990) and cortex (Squire, 1986). LTP in hippocampus has been used as a dominant model of the activity-dependent synaptic plasticity in the mammalian brain. In 1949, Hebb proposed a coincidence-detection rule in which synaptic modification can occur only when presynaptic and postsynaptic activity are concomitant. The NMDA receptor has all the important characteristics to be the molecular coincidence detector. The presynaptic activity is required to provide the NMDA agonist (glutamate), and postsynaptic depolarization is required to reduce the Mg^{2+} blockade of NMDA receptor channels (reviewed in Collingridge and Singer, 1990). These two events are required for the NMDA receptor to open and thus to trigger the induction of LTP. The subsequent maintenance of LTP involves an increase in presynaptic transmitter release. It is thought that a retrograde messenger is involved in transfer of information from the postsynaptic cells to the presynaptic terminal where LTP is maintained (reviewed in Kandel and O'Dell, 1992).

1.6.2 GLUTAMATE NEUROTOXICITY

Although glutamate is a neurotransmitter at the majority of excitatory synapses, its role as a cytotoxin was also proposed. In the late 1960s, Olney proposed excitotoxic hypothesis of cell death and involvement of glutamate and some other excitatory amino acids (Olney and Sharpe, 1969; Olney and Ho, 1970). It has been demonstrated that systematic administration of glutamate into different brain region, caused characteristic cytopathology in which postsynaptic cells were destroyed but presynaptic terminals and non-neuronal cells survived. With the availability of different agonists and antagonists of glutamate receptors, it was possible to study the role of glutamate ionotropic receptors, NMDA, AMPA and kainate in a neurotoxicity (Watkins *et al.*, 1990). It was shown in the brain that different agonists could selectively kill different populations of neurons

and in some cases this could reflect the pattern of receptor expression (Meldrum and Garthwaite, 1990). As the study of glutamate neurotoxicity *in vivo* was difficult because of high glutamate uptake mechanisms, different approaches were developed to study glutamate toxicity *in vitro*. Using different antagonists of glutamate receptors in studying toxicity in tissue culture, results indicated that NMDA receptors were mediators of neurotoxicity in some cultures (Schramm *et al.*, 1990; Lombardi *et al.*, 1993), non-NMDA receptors in others (Mattson *et al.*, 1989; Frandsen *et al.*, 1990), while both subtypes were involved in some other cultures (Schousboe, *et al.*, 1992; Sucher *et al.*, 1991; Frandsen and Schousboe, 1993). It was suggested that the disturbance of Ca^{2+} homeostasis was a major contributing factor to neurotoxicity induced by excitatory amino acids (Tymianski *et al.*, 1993; Frandsen and Schousboe, 1993; Milani *et al.*, 1991). Prolonged exposure of most cultured neurons to micromolar concentrations of glutamate led to two types of neurotoxicity, rapid and delayed, although it seems that they had a common toxic mechanism, excessive Ca^{2+} influx. Release of Ca^{2+} from internal stores also contributed to neurotoxicity induced by NMDA, glutamate or quisqualate but not induced by AMPA or kainate (Frandsen and Schousboe, 1993).

NMDA Receptor-mediated neurotoxicity was the most well characterized (Chuang *et al.*, 1992). Rapid NMDA toxicity was prevented only when the antagonist was administered during agonist exposure (Choi, 1988; Chen *et al.*, 1992). Delayed NMDA toxicity was inhibited by administration of NMDA receptor antagonists even several hours after agonist induction. Delayed NMDA toxicity could also be prevented by preincubation with subtoxic concentrations of NMDA, and this protective NMDA effect required *de novo* RNA and protein synthesis (Chuang *et al.*, 1992; Marini and Paul, 1992). Neurotoxicity elicited by glutamate activation of NMDA receptors could be potentiated by polyamines, and furthermore the antagonist of the polyamine site, ifenprodil, was shown to have neuroprotective effect against it (Shalaby *et al.*, 1992; Lombardi *et al.*,

1993). It is interesting that developmental expression of GBP (1.4.1) immunoreactivity closely paralleled the expression of sensitivity to NMDA neurotoxicity (Mattson *et al.*, 1991). Non-NMDA neurotoxicity can be AMPA receptor-mediated and kainate receptor-mediated. Neurotoxicity induced by kainate has been well documented (e.g. Olney *et al.*, 1974; Kato *et al.*, 1991). For acute and delayed kainate neurotoxicity, two different mechanisms were proposed (Kato *et al.*, 1991). The time course of delayed kainate neurotoxicity was similar to NMDA mediated neurotoxicity. It has been reported that kainate neurotoxicity was age related, with older animals more sensitive to kainate than younger animals (Wozniak *et al.*, 1991). Glutamate, NMDA and kainate induced neurotoxicity could also be attenuated by activation of the metabotropic glutamate receptor (Pizzi *et al.*, 1993; Koh *et al.*, 1991).

Glutamate neurotoxicity may be involved in both acute and chronic neural degeneration. The acute degenerative changes with excitotoxic mechanisms occurs after four types of acute injury to the CNS; cerebral ischaemia, hypoglycaemia, status epilepticus, and trauma (Meldrum, 1991, Meldrum, 1990, Hosford *et al.*, 1991). Studies with NMDA receptor antagonists showed dramatic protection following middle cerebral artery occlusion in adult mice, neonatal anoxia-ischaemia in rats and rabbits and incomplete global ischaemia. NMDA receptor antagonists were not protective against complete global ischaemia, but the non-NMDA receptor antagonist NBQX was protective against complete global ischaemia even when administered with significant post-ischaemic delay (Sheardown *et al.*, 1990). It was observed that during transient forebrain ischaemia, expression of GluR2 subunits was reduced in the CA1 hippocampal neurons which coincided with the increase in Ca^{2+} influx into the same neurons at a time point that preceded their degeneration, suggesting an important role for the switch in GluR2 subunit expression in post-ischaemic cell death (Pellegrini-Giampietro *et al.*, 1992). Domoate (analog of kainate) was believed to be responsible for seafood poisoning that caused acute sensory-motor disturbances

and chronic memory impairment in some victims, after eating mussels that have fed on seaweed (e.g. Tasker *et al.*, 1991). The chronic neurodegenerative disorders, in which glutamate excitotoxicity has been implicated include Huntington's disease, Alzheimer type of senile dementia, Parkinsonism, olivopontocerebellar atrophies and amyotrophic lateral sclerosis (e.g. Krogsgaard-Larsen *et al.*, 1992; Rothstein *et al.*, 1993; Dure *et al.*, 1991). There was also evidence for the possible importance of glutamate in the pathophysiology of schizophrenia (Kerwin *et al.*, 1990; Reynolds, 1992).

1.7 THE OBJECTIVES OF THIS STUDY

L-Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. Molecular cloning has identified five genes encoding the subunits of the NMDA subclass of the glutamate receptor, NMDAR1 and NMDAR2A-2D. The main aim of this thesis was to establish the transient expression of these respective subunits in mammalian cells as a model system for structure-function studies of the recombinant NMDA receptor complex. Firstly, the subcloning of cDNAs encoding the NMDAR1-1a and NMDAR2A subunits in a mammalian expression vector was described. Secondly, optimal conditions were established for the transient expression of homomeric and heteromeric NMDA receptors. As the co-expression of the NMDAR1-1a and NMDAR2A subunits resulted in cell death, a study was performed to determine the ability of a series of different NMDA antagonists in the prevention of cell death. Site-directed *in vitro* mutagenesis was also used in attempt to identify residues in channel-forming TM2 domain, important for the NMDA-receptor mediated cell toxicity and Mg²⁺-blockade of the NMDA receptor.

CHAPTER 2

Transient expression of NMDA heteromeric receptors in mammalian cells

2. 1 INTRODUCTION

2.1.1 EXPRESSION OF GENES IN MAMMALIAN CELLS

There are two main groups of expression systems, prokaryotic and eukaryotic. The choice of system depends on the purpose for which the protein is being expressed and on the class of protein. If the protein does not require post-translational modification, a prokaryotic system, such as expression in the bacterium Escherichia coli (E. coli), can be used. When post-translational modification is important, eukaryotic expression systems are the choice. Different eukaryotic systems include expression in yeast cells, insect cells or mammalian cells. These systems can be divided into two main groups: those that involve the transient or stable expression of transfected DNA (direct DNA transfer) and those that are mediated by virus infection, such as Simian virus 40 (SV40), vaccinia virus, adenovirus, retrovirus or baculovirus (reviewed in Goeddel, 1990). The direct DNA transfer into cells can be either transient or alternatively the DNA can be stably integrated into the host cell chromosomal DNA. Stable expression is obviously more advantageous than transient expression systems but it can be labour intensive; therefore, many studies of the characterisation of receptor proteins have employed the transient system. The efficiency of expression of proteins in both transient and stable systems is cell line and vector dependent. The following factors are important for efficient transient expression system, the number of cells which take up a DNA, the gene copy number per cell, the gene mRNA processing and stability, transcriptional and translational efficiency of the gene. Both genomic DNA and cDNA can be used for direct DNA transfer.

2.1.2 CELL LINES FOR EXPRESSION OF cDNAs

There are many cell lines which have been established for the expression of different classes of proteins. Two cell lines most frequently used for the transient expression of membrane-bound receptors are the COS cell line and the HEK 293 cell line. COS cells are derived from African green monkey kidney CV-1 cells by transformation with an origin-defective SV-40 mutant virus. These COS cells express high levels of the SV-40 large-tumour (T) antigen which is required to initiate viral DNA replication to greater than 10 000 copies per cell and they also contain all of the cellular factors required to drive the replication of SV-40 origin containing plasmids (Gluzman, 1981). The COS cell-based expression systems were used extensively in studying the function of β -adrenergic receptors (e.g. Strader *et al.*, 1987) and nicotinic acetylcholine receptors (Verrall and Hall, 1992). The HEK 293 cell line is adenovirus transformed and the cells constitutively express adenovirus E1a protein (Gorman *et al.*, 1989). In the presence of the adenovirus early region protein E1a, inhibition of SV-40 and Rous sarcoma virus (RSV) promoters, through enhancer repression has been observed. In the same cell line, activity of the human cytomegalovirus (CMV) promoter was enhanced. Based on that observation, an expression system which consists of HEK 293 cells and a CMV driven vector has been used to produce sufficient quantities of test protein shortly after transfection, for example human growth factor (Gorman *et al.*, 1990), and membrane bound proteins, such as GABA_A receptors (Pritchett *et al.*, 1988), and glutamate receptors (Sommer *et al.*, 1990). Such transient expression systems require specially designed vectors which are able to carry foreign DNA and to replicate in both bacterial and mammalian cells.

2.1.3 FUNCTIONAL COMPONENTS OF MAMMALIAN EXPRESSION VECTORS

Most of the mammalian expression vectors in current use contain prokaryotic sequences that facilitate the propagation of the vector in bacteria and multiple elements that are expressed only in eukaryotic cells. Prokaryotic sequences include the origin of replication (ColE1) for any *E. coli* strain, the M13 origin of replication for the rescue of single-stranded DNA (ssDNA), the T7 and Sp6 RNA promoters flanking a multiple cloning site for *in vitro* transcription of sense and antisense RNA and a gene encoding antibiotic resistance e.g. genes for ampicillin, kanamycin and tetracyclin resistance, to permit selection of bacteria which harbour the plasmid DNA. Multiple elements consist of non-coding and coding sequences and include: an origin of replication for the vector amplification in mammalian cells e.g. SV40 or Epstein Barr virus, an efficient promoter and enhancer elements, mRNA processing signals that include splicing and polyadenylation sequences and selectable markers that can be used to select mammalian cells that have stably-integrated the plasmid DNA.

2.1.3.1 Promoter and enhancer elements

Two important DNA sequences that have been identified to interact specifically with cellular proteins involved in transcription are the promoter and enhancer sequences. There are two different types of promoters, the inducible promoter and the constitutive promoter. The latter is unregulated whereas the former is regulated by external stimulus and can be used to express potentially cytotoxic protein, an example is the mammary tumour virus LTR promoter, which is glucocorticoid-inducible. In higher eukaryotes, constitutive promoters contain two types of motifs: an AT-rich DNA sequence motif, the so-called

TATA box which is an essential promoter component about 20-25 base pairs (bp) upstream of the transcriptional initiation site. An additional type of sequence motif is an upstream element, the CAAT box which is located 80 bp upstream from the mRNA initiating site. Enhancers are activators of gene transcription in orientation- and distance-independent manner and they were the first DNA sequences found to be tissue-specific (reviewed in Maniatis *et al.*, 1987), although some of them are very active in a wide variety of cell types, such as SV40 (reviewed in McKnight *et al.*, 1986), RSV and CMV enhancers (Boshart *et al.*, 1985).

2.1.3.2 mRNA processing signals

Transcription termination, site-specific post-translational cleavage and polyadenylation are essential steps in the 3' end of mRNA processing and these steps are important for mRNA stability. Two sequences important for efficient polyadenylation have been found. The first is a GU- or U-rich sequence located downstream from the polyadenylation signal and the second one is a highly conserved hexanucleotide, AAUAAA located 11-30 nucleotides upstream of most polyadenylation signals. The most frequently used cleavage-polyadenylation signals in mammalian expression vectors are those derived from the SV40 virus.

2.1.4 EXAMPLES OF VECTORS FOR EXPRESSION OF cDNAs ENCODING EUKARYOTIC GENES IN MAMMALIAN CELLS

Most vectors in current use contain elements that are useful for expression in a variety of cell lines of different origin, such as the viral, SV40 and CMV promoter/enhancer elements, therefore enabling their wide use. An attempt has

been made to utilize mammalian promoters active in different types of tissues to drive expression of mammalian genes, an example is the β -actin promoter driven vector, pH β APr-1-neo.

pSVK3. The plasmid pSVK3 was constructed by Mongkolsuk (1988). It contains SV40 early promoter/enhancer elements that function efficiently in a wide variety of mammalian cells. The SVK3 contains also an SV40 origin of replication and mRNA processing signals for replication and expression in eukaryotic cells. This vector has the M13 origin of replication for producing ssDNA upon infection of bacterial culture with the helper phage M13KO7 (Vieira and Messing, 1987). The ColE1 origin of replication permits efficient replication in E. coli cells and the T7 RNA polymerase promoter enables *in vitro* transcription of cloned genes.

pH β APr-1-neo. This is a β -actin promoter driven vector that consists of the human β -actin gene 5' flanking sequence plus a 5' untranslated region and intervening sequence 1, followed by a SV40 polyadenylation signal (Gunning et al., 1987). The plasmid has a pBR322 origin of replication and an ampicillin gene for propagation in E. coli and selection of bacterial cells carrying the plasmid DNA. The neomycin-resistance gene is included in the vector to allow selection of mammalian cells that stably integrate this vector into their genome. Promoter activity of this vector was tested in different human and rodent cell lines and was comparable to that of SV40 early promoter.

pCDM8. The plasmid pCDM8 is a CMV promoter-enhancer driven vector. The T7 RNA promoter is flanking the multiple cloning site for *in vitro* transcription of sense RNA. The pCDM8 contains SV40 processing signals for mRNA stability, SV40 and polyoma origins of replication for replication in eukaryotic cells. The ColE1 origin and supF suppressor tRNA are included to permit maintenance in E. coli strains with P3 episome containing genes for kanamycin, amber tetracycline and amber ampicillin resistance, such as the

MC1061/P3 strain. ssDNA rescue is possible by using the M13 origin of replication. The plasmid pCDM8 has been used together with HEK 293 cells as an expression system for expression of membrane-bound receptor proteins such as the GABA_A receptor (Kusama *et al.*, 1993) and non-NMDA glutamate receptors (Keinänen *et al.*, 1990).

pCIS. The plasmid pCIS is a CMV promoter-enhancer driven vector constructed by Gorman *et al.*, (1990), (Figure 3.3.2). It has proved useful for the expression in HEK 293 cells of secreted proteins such as relaxin, nerve growth factor and membrane-bound proteins, such as GABA_A receptors (Pritchett *et al.*, 1988), glycine receptors (Pribilla *et al.*, 1992) and non-NMDA glutamate receptors (Sommer *et al.*, 1992). The pCIS contains SV40 elements, such as mRNA processing signals and origin of replication. It is readily propagated in *E. coli* cells because of the ColE1 origin of replication. The ampicillin-resistance gene permits selection of bacterial colonies carrying plasmid DNA. The M13 origin of replication is included in pCIS for ssDNA rescue (+, sense strand), upon superinfection of bacterial culture with helper phage M13KO7 (Vieira and Messing, 1987).

2.1.5 METHODS FOR INTRODUCING DNA INTO MAMMALIAN CELLS

For the efficient expression of protein in mammalian cells, an important factor is the percentage of cells which actually take up the plasmid DNA. Several methods have been developed for introducing the plasmid DNA into different mammalian cell lines. They include: calcium phosphate-mediated transfection (e.g. Gorman *et al.*, 1990), diethylaminoethyl-dextran- (DEAE-dextran, e.g. Levesque *et al.*, 1991) mediated transfection and polybrene mediated-transfection (e.g. Helgen and Fallon, 1990), electroporation (e.g. Chu

et al., 1987) and lipofection (e.g. Ferroni et al., 1992).

Perhaps the most widely used transfection techniques is the calcium phosphate-mediated transfection. The calcium phosphate-DNA coprecipitate is formed after mixing DNA with calcium phosphate and this is then presented to cells. It is believed that the calcium phosphate-DNA coprecipitate enters the cell cytoplasm by endocytosis and it is then transferred to the nucleus (Loyter et al., 1982). The transfection efficiency is cell type-dependent and can be increased by treatment of the cells by glycerol (Gorman et al., 1990) or chloroquine (Luthman and Magnusson, 1983). A highly efficient modification of the calcium phosphate-mediated transfection has been described by Chen and Okayama, (1987). In their method, the calcium phosphate-DNA precipitate is allowed to form as the cells are incubated for 15-24 h under controlled conditions of pH 6.96 and a CO₂ contents of 2-4 %. The DEAE-dextran mediated DNA transfection was originally used to introduce SV40 and polyoma virus DNAs and has been found to work well only for transient expression in cell lines such as CV-1 and COS (e.g. Verrall and Hall, 1992). A new method using a synthetic cationic lipid such as Transfectam has been recently developed (Behr et al., 1989). This molecule has lipo-spermine headgroup that interact strongly with the DNA, coating it with a cationic lipid layer, thus promoting the binding to cell membrane. Transfectam is a non-toxic molecule and in comparative studies it has been shown to give higher efficiency of transfection than those obtained with calcium phosphate- and DEAE-dextran-mediated transfection.

In this Chapter, the establishment of a transient expression in HEK 293 cells of the NMDA receptors will be described.

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Restriction enzymes, T4 DNA ligase, DNA molecular weight markers II (λ DNA Hind III digested), calf intestinal alkaline phosphatase (CIP), lysozyme, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (x-gal), ampicillin and caesium chloride (CsCl) were obtained from Boehringer Mannheim GmbH (Lewes, East Sussex, UK). The plasmid pSV- β -galactosidase, Cyto Tox 96TM Non-Radioactive Cytotoxicity Assay and MagicTM DNA clean-up system were purchased from Promega Ltd. (Southampton, UK). Agarose (Ultra Pure) and low melting point (LMP) agarose were purchased from Life Technologies Limited (Uxbridge, UK). QIAEX, gel extraction kit was purchased from Hybaid Limited (Middlesex, UK). Microbiological culture media (tryptone, agar bacto and tryptone yeast extract broth) were obtained from Difco Laboratories Limited (East Molesey, UK). Phenol (pre-equilibrated, pH > 7.6) was from Fisons (Leicestershire, UK). Tissue culture media (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham, DME/F-12 1:1 mixture), trypsin-EDTA, Hanks' balanced salt solution (HBSS), trypan blue stain, poly-L-lysine, polyethylene glycol (PEG, av. mol. wt. 8000), ribonuclease A (RNase A), tunicamycin and ethidium bromide were obtained from Sigma (Poole, Dorset, UK). Sterile foetal calf serum (FCS) was obtained from Advanced Protein Products Ltd. (West Midlands, UK). Sterile disposable filter units Sartolab-V150 were from Sartorius (Goettingen, Germany). CELLocate microgrid (55 μ m) coverslips from Eppendorf were obtained from Biotech Instruments Ltd. (Luton, UK). DL-2-amino-5-phosphonopentanoic acid (AP5), 5,7-dichlorokynurenic acid (DKA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Neuramin (Essex, UK). All other materials were from commercial sources.

Gifts: The plasmid pCIS was a gift from Dr. C. Gorman, Genetech, San Francisco, California, USA. The plasmid pN60 with the cDNA encoding the full length rat NMDAR1-1a subunit was the generous gift from Dr. Shigetada Nakanishi, The Faculty of Medicine, Kyoto University, Kyoto, Japan. The plasmid pBKA ϵ 1 with the cDNA encoding the full length mouse ϵ 1 (NMDAR2A), subunit was the generous gift from Dr. Masayoshi Mishina, The School of Medicine, Niigata University, Niigata 951, Japan. HEK 293 cells were a gift from Dr. T.G. Smart, The School of Pharmacy, London, UK. Chinese Hamster Ovary (CHO) cells were a gift from Dr. P.G. Strange, The University of Kent, Canterbury, UK.

Strain: (*E. coli*) strain INV1 α (Genotype: endA1, recA1, hsdR17(r_k^- , m_k^+), supE44, λ^- , thi-1, gyrA, relA1, Φ 80lac Δ ZM15 Δ (lacZYA-argF), deoR $^+$, F $^-$) was obtained from British Bio-technology Limited (Oxford, UK).

2.2.2 STORAGE OF THE E. COLI CELLS

For long term storage, 0.5 ml of overnight bacterial cultures were aliquoted into freezing vials and mixed with 0.5 ml medium containing tryptone 10 g/l, yeast extract broth 5 g/l, and NaCl 10 g/l, (LB medium), supplemented with 50 % (v/v) sterile glycerol and 35 μ g/ml ampicillin. The vials were stored at -20°C.

2.2.3 PREPARATION OF COMPETENT E. COLI CELLS

The competent *E. coli* cells (INV1 α) were prepared as described in Nishimura et al., (1990). A 50 ml culture was inoculated with 0.5 ml overnight culture and grown in an orbital Luckham R300 shaker, at 200 rpm, 37°C in LB medium (2.2.2) supplemented with 10 mM MgSO $_4$ and 0.2 % (w/v) glucose (medium A), to mid-logarithmic phase, i.e. OD $_{\lambda=600\text{ nm}}$ = 0.6-0.8. The cells were

kept on ice for 10 min and then pelleted at 300 x g for 10 min at 4°C. The cells were resuspended gently using a sealed Pasteur pipette in 0.5 ml ice cold medium A then, 2.5 ml storage solution B (LB medium supplemented with 36 % (v/v) glycerol, 12 % (w/v) polyethylene glycol (PEG, MW 8000) and 12 mM MgSO₄), was added and mixed well using a sealed Pasteur pipette. The competent cells were divided into aliquots of 0.1 ml in pre-cooled 1.5 ml Eppendorf tubes and stored at -80°C until use. The cells could be stored at -80°C for a period of up to three months without losing transformation efficiency.

2.2.4 TRANSFORMATION OF COMPETENT E. COLI CELLS

For transformation, the frozen cells were thawed on ice, mixed immediately with 2-5 µl (10-50 ng) of plasmid DNA and incubated on ice for 30 min. The cells were transferred to a water bath and incubated for 60 sec at 42°C, chilled on ice for 1-2 min, followed by a 10-fold dilution into prewarmed medium A (2.2.3) and incubated at 37°C for 60 min. Samples 50-300 µl were plated on LB agar plates (LB medium containing 1.5 % (w/v) agar and 70 µg/ml ampicillin). Plates were incubated for 15-18 h at 37°C. Following incubation, colonies were counted and overnight cultures prepared for small-scale plasmid preparations as described in 2.2.5.

2.2.5 PREPARATION OF PLASMID DNA

2.2.5.1 Small-scale preparation of plasmid DNA

A single colony of E. coli containing the plasmid, was transferred from the LB plate stock to 10 ml LB medium (2.2.2) containing 100 µg/ml ampicillin in a loosely capped 50 ml Falcon tube. The culture was incubated overnight at

37°C with shaking (200 rpm). The overnight culture (1.5 ml) was centrifuged at 12 000 x g for 3 min at room temperature. The supernatant was removed by aspiration and the pellet re-centrifuged for 30 sec and re-aspirated. The pellet was resuspended in 100 µl ice-cold 50 mM Tris-HCl, pH 8.0, containing 50 mM glucose and 10 mM EDTA (TGE buffer), by pipette suction. To each tube, 200 µl of freshly prepared 200 mM NaOH and 1 % (w/v) SDS (alkaline sodium dodecyl sulphate (SDS) solution), was added and the contents were mixed by inverting the tube rapidly five times. Tubes were stored on ice. To each, 150 µl of ice-cold 3 M potassium acetate, pH 4.8, was added and the tube was mixed by inversion. It was kept on ice for 5 min, centrifuged at 12 000 x g for 10 min at 4°C and the supernatant was transferred to a fresh tube.

The supernatant from the previous step, was extracted with an equal volume of phenol:chloroform mixture (phenol:chloroform:amyl alcohol, 25:24:1, volume ratio) and centrifuged at 12 000 x g for 1 min at room temperature. The upper layer was transferred to a fresh tube and re-extracted with one volume of chloroform.

The double-stranded plasmid DNA was precipitated with 2 volumes of absolute ethanol for 15 min on ice and pelleted at 12 000 x g for 10 min at 4°C. The supernatant was removed and the pellet was washed with 1 ml 70 % (v/v) ethanol. The plasmid DNA pellet was dried in a vacuum desiccator for 15 min and dissolved in 50 µl of 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA (TE buffer) and RNase (20 µg/ml). For routine DNA precipitation, the salt concentration was adjusted by adding 1/10 of 3 M sodium acetate, pH 5.2 and DNA was precipitated as above. RNase containing TE buffer was used only for small scale plasmid preparations.

For some plasmid preparations this method was scaled up by growing cells in 50 ml of Terrific Broth media (2.2.5.4). Other solutions were scaled up appropriately.

2.2.5.2 Large-scale preparation of plasmid DNA

For large scale plasmid preparations the bacterial growth medium was Terrific Broth. This was prepared by dissolving tryptone 12 g/l, yeast extract broth 24 g/l and glycerol 4 ml/l in sterile water and after autoclaving and cooling below 60°C, the medium was supplemented with 100 ml/l of sterile buffer which was 0.17 mM KH_2PO_4 and 0.72 mM K_2HPO_4 . Terrific Broth medium (200 ml), containing 70 µg/ml ampicillin was inoculated with 1 ml of overnight culture in a 500 ml flask. The culture was incubated for 14-18 h at 37°C with shaking at 300 cycles per minute on a rotary shaker. The culture was transferred into two ice-cold JA-14 centrifuge tubes, kept on ice for 10 min and centrifuged at 20 000 x g for 10 min at 4°C in the Beckman J2-21 centrifuge. The supernatant was discarded and the tubes inverted on paper tissue to allow the supernatant to drain away. Each bacterial pellet was resuspended in 2.5 ml ice-cold TGE buffer (2.2.5.1) and transferred into a pre-cooled JA-20 tube. TGE buffer containing 10 mg/ml of freshly prepared lysozyme (2.5 ml) was added and the tubes were incubated for 5 min at room temperature. They were placed on ice, 10 ml freshly prepared alkaline SDS (2.2.5.1) was added and the contents mixed by inverting the tubes several times. After 10 min incubation on ice, 6 ml ice-cold 3 M potassium acetate solution, pH 4.8, was added and the contents mixed by shaking the tubes several times. The tubes were stored on ice for 10 min and centrifuged at 30 000 x g for 35 min at 4°C. The supernatants were transferred into fresh JA-20 tubes, each mixed with 12.5 ml propan-2-ol and stored at room temperature for 15 min. The tubes were centrifuged at 10 000 x g for 20 min at room temperature, the supernatant was removed and the DNA pellet washed with 70 % (v/v) ethanol. Each plasmid DNA pellet was resuspended in 4 ml TE buffer (2.2.5.2) and stored at 4°C.

CsCl (4 g) was added to the plasmid DNA from the large scale plasmid preparation as above (4 ml), and mixed gently until dissolved. Ethidium bromide

(EtBr, 200 μ l, 10 mg/ml in water) was added and immediately mixed followed by centrifugation at 10 000 x g for 20 min at 4°C. The supernatants were transferred into the Beckman Quick-seal tubes using a 2 ml disposable syringe fitted with a 21 gauge needle. Centrifugation was carried out using the vertical VTi 65 rotor at 400 000 x g for 16 h at 24°C in the Beckman L8-60M ultracentrifuge. The band which was the closed circular plasmid DNA was collected by using a disposable 1 ml syringe fitted with a 21 gauge needle and transferred to a fresh microfuge tube. To the DNA solution, an equal volume of butan-1-ol was added and two phases were mixed by shaking the tubes. The mixture was centrifuged at 10 000 x g for 30 sec at room temperature and the upper phase was discarded. Extraction was repeated 4-5 times until all the pink colour of the ethidium bromide had disappeared. The aqueous phase was dialysed for 24-48 h against several changes of TE buffer, pH 8.0, to remove CsCl. Finally, the DNA concentration was determined as in 2.2.7 and the DNA solution stored in aliquots at 4°C for short term and at -20°C for long term storage.

When the DNA was purified to be used for HEK 293 mammalian cell transfection, two rounds of CsCl density gradient centrifugation were carried out. The band which was the closed circular plasmid DNA was collected after the first CsCl centrifugation. CsCl and EtBr were added as above and a second centrifugation was carried out at 400 000 x g for 16 h at 24°C.

2.2.6 SPECTROPHOTOMETRIC QUANTIFICATION OF PLASMID DNA

Optical density measurements at $\lambda = 260$ nm and $\lambda = 280$ nm ($OD_{\lambda=260, 280}$) were used to calculate DNA concentration and purity (Sambrook *et al.*, 1989). The concentration was calculated as follows: an $OD_{\lambda=260} = 1$ corresponds to approximately 50 μ g/ml for dsDNA, 40 μ g/ml for ssDNA and 20 μ g/ml for

oligonucleotides. The ratio between the readings at $\lambda = 260$ nm and $\lambda = 280$ nm ($OD_{\lambda=260}/OD_{\lambda=280}$) gives an estimate of the purity of the nucleic acid. Pure preparations of DNAs have $OD_{\lambda=260}/OD_{\lambda=280}$ values of 1.8.

2.2.7 RESTRICTION ENZYME DIGESTION

The following protocol was for a typical reaction containing 1-2 μ g plasmid DNA. For larger amounts of DNA, the reaction was scaled appropriately. DNA solution was placed in a sterile tube and mixed with sterile water to give a volume of 17 μ l. The appropriate 10 x restriction enzyme buffer (2 μ l), supplied by the manufacturer, e.g. buffer H for *Eco RI* restriction enzyme, was added and mixed gently. The restriction enzyme (5-10 units, 0.5-1 μ l) was added, mixed and briefly centrifuged. The mixture was incubated at 37°C for 1-2 h. The enzyme was deactivated by heating the mixture at 70°C for 10 min. An aliquot (1 μ l) was analysed by flat bed agarose gel electrophoresis as described in 2.2.13.

2.2.8 DEPHOSPHORYLATION OF LINEARIZED PLASMID DNA

The plasmid DNA (7-12 μ g) was digested as described above and purified using MagicTM clean-up resin (2.2.10). To the purified plasmid DNA (30 μ l), 10 x buffer containing 100 mM Tris-HCl, pH 8.3, 10 mM MgCl₂, 4 μ l, and 10 mM ZnCl₂, 4 μ l, were added and mixed. Calf intestinal alkaline phosphatase (CIP) was added (1.5 units, 1.5 μ l) and the mixture was incubated at 37°C for 30 min. CIP was deactivated by heating at 70°C for 10 min and removed using MagicTM clean-up resin.

2.2.9 DNA LIGATION REACTIONS

Typical ligation reactions were carried out in a 20 μ l volume at 4°C. The plasmid DNAs were cut with the appropriate restriction enzymes and if required, linearised plasmid DNA was dephosphorylated with CIP (2.2.8). Desired fragments were isolated by flat bed agarose gel electrophoresis and purified by QIAEX resin (2.2.13). In a sterile microfuge tube, vector DNA (150 ng) was mixed with an equimolar amount of foreign DNA in a total volume of 10 μ l. The ligase mix was made by mixing T4 DNA ligase (2 units, 2 μ l), 10 x buffer containing 660 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 10 mM dithiothreitol, 10 mM ATP, 2 μ l and water 6 μ l. This ligase mix was added to DNAs mixture. The contents were mixed gently and incubated at 4°C for 16-20 h. The enzyme was deactivated by heating at 70°C for 10 min and an aliquot was removed for transformation of competent *E. coli* cells as described in 2.2.4.

When low melting point (LMP) agarose was used for flat bed gel electrophoresis (2.2.12.3), the ligation reaction was modified as follows, DNA fragments, generated by restriction enzyme digestion (2.2.7) were separated on a LMP agarose gel and cut out as described in 2.2.12.3. The melted gel slices were mixed in prewarmed Eppendorf tube. Vector DNA (150 ng) was mixed with equimolar amount of foreign DNA in a total volume of 10 μ l and incubated for 5 min at 37°C. Ligation mix, prepared as above, was added to the tube, mixed and incubated at 4°C for 20-24 h.

2.2.10 MAGIC™ DNA PURIFICATION

Magic™ Clean-up resin was used as recommended by the manufacturer to purify plasmid DNA after dephosphorylation with CIP and after restriction enzyme digestion. Clean-up resin (1 ml) was mixed with 50-500 μ l of sample by gentle inversion. The clean-up resin containing the bound DNA was pipetted into

a syringe barrel attached to a minicolumn and the slurry was pushed gently into the minicolumn. The minicolumn was washed by pushing through 2 ml 80 % (v/v) isopropanol and dried by centrifugation in a 1.5 ml microfuge tube at 12 000 x g at room temperature. The minicolumn was transferred to a fresh microfuge tube and 30-50 µl prewarmed (65-70°C) TE buffer, pH 7.6, was applied. After 1 min incubation at room temperature, the microcolumn was centrifuged at 12 000 x g for 20 sec to elute the bound DNA. The purified DNA was stored at 4°C.

2.2.11 PREPARATION OF DIALYSIS TUBING

Dialysis tubing were prepared as in Sambrook *et al.*, (1989). The tubing, visking size 1-8/32" was cut into pieces 10 cm in length and boiled for 10 min in a large volume of 2 % (w/v) sodium bicarbonate and 1 mM EDTA, pH 8.0. After rinsing in distilled water, the tubing was boiled for 10 min in 1 mM EDTA, pH 8.0. Finally, the tubing was allowed to cool, put into an sterile bottle, rinsed with sterile water and stored at 4°C in 50 % (v/v) of ethanol and 10 mM EDTA, pH 8.0. The tubing was always submerged in storing solution and before use, rinsed in sterile water.

2.2.12 FLAT BED AGAROSE GEL ELECTROPHORESIS

2.2.12.1 The agarose gel

Agarose gels were prepared according to Sambrook *et al.*, (1989). All electrophoretic analyses were performed using a Flowgen minigel apparatus MH 1070. The following stock solutions were prepared:

(a) 5 x Tris-borate running gel buffer 0.45 M Tris buffer, pH 8.0, 0.45 M boric acid and 20 mM EDTA (TBE buffer).

(b) 6 x gel-loading buffer 0.25 % (w/v) bromphenol blue, 30 % (v/v) glycerol and 60 mM EDTA, pH 8.0.

(c) 0.9 % (w/v) agarose in 1 x TBE buffer. This stock solution was stored at 60°C after autoclaving.

To make a 0.9 % (w/v) agarose/EtBr gel, 40 ml of the agarose stock solution was precooled to 40-50°C, mixed with 5 µl of EtBr (10mg/ml) and immediately poured into the gel apparatus assembled with the aluminium casting gates and the well-forming comb. The gel was allowed to polymerize for at least 30 min at room temperature. It was overlaid with 1 x TBE running buffer to a depth of about 2-3 mm over the gel. DNA samples were prepared as follows: DNA (about 50-150 ng) was mixed with 2 µl of 6 x gel-loading buffer and water was added to a final volume of 12 µl. The mixture was loaded slowly into the slots. Electrophoresis was carried out at 50 V constant voltage for 30-60 min and the gel was examined by Flowgen UV Transilluminator (TF-10M), at $\lambda = 312$ nm.

Agarose gels were photographed under UV illumination using a Polaroid DS34 instant camera fitted with a Wratten 22A filter (30 sec at f 11) and Polaroid 665 positive/negative instant film. Negatives were cleared in 18 % (w/v) sodium sulphite solution for 60 sec, washed and stored at room temperature.

2.2.12.2 The LMP flat bed agarose gel electrophoresis

When DNA was to be used in ligation reactions without extraction from the gel, specially purified LMP agarose was used. The gel was cast with LMP agarose (0.9 %, w/v) and a well-forming comb (50 µl). To pour and run a gel, 40 mM Tris-acetate, pH 8.0, 1 mM EDTA (1 x TAE buffer), was used. The gel was allowed to harden for 30 min at room temperature and for 30 min at 4°C. Gel electrophoresis was carried out for 60 min at 30 V constant voltage at 4°C. The desired bands were identified under UV illumination and cut out in the

minimum volume, 50-100 μ l, under UV illumination. The melted gel slices (70°C, 15 min) were used directly in the ligation reaction (2.2.9).

2.2.13 QIAEX AGAROSE GEL EXTRACTION

Flat bed agarose gel electrophoresis was carried out as described (2.2.12), and the DNA band of interest was excised under UV illumination. The gel slice was placed into a 1.5 ml microfuge tube and the slice weight was determined. Per 100 mg of gel, 300 μ l of solubilization buffer 10 mM Tris-HCl, pH 7.0, 3 M NaI, 4 M NaClO₄, 10 mM sodium thiosulphate (QX1 buffer) and 0.1 volume 1 M mannitol were added and the tube was incubated at 50°C for 10 min. To the solubilised gel solution, 10 μ l of QIAEX was added and the mixture was incubated at 50°C for 10 min. During that time, the sample was vortexed briefly every 2 min. The sample, which contained DNA bound to QIAEX was centrifuged for 30 sec and the supernatant was removed. The pellet was resuspended in 500 μ l of 10 mM Tris-HCl, pH 7.0, 8 M NaClO₄, (QX2 buffer) by vortexing, centrifuged for 30 sec and the supernatant was discarded. This washing step was repeated once. The pellet was resuspended in 500 μ l of 10 mM Tris-HCl, pH 7.5, 70 % (v/v) ethanol, 100 mM NaCl, (QX3 buffer), by vortexing, centrifuged for 30 sec and the supernatant was removed. This step was repeated, traces of ethanol were carefully removed and the pellet was dried in a vacuum desiccator for 5 min. To elute DNA from the QIAEX resin, 20 μ l TE, pH 8.0, was added. The pellet was resuspended by vortexing and kept for 5 min at room temperature. The suspension was centrifuged for 30 sec at 10 000 x g and the supernatant containing clean DNA was removed into a fresh tube.

2.2.14 PREPARATION OF CELL CULTURE MEDIA

Powdered DME/F-12 1:1 mixture was dissolved in 800 ml sterile water and supplemented with 100 ml of foetal calf serum (FCS), 20 ml penicillin/streptomycin antibiotic (final: penicillin 500 IU/ml, streptomycin 500 µg/ml) and 40 ml 7.5 % (w/v) NaHCO₃ solution (final 3.0 g/l). Using 2 M NaOH, the pH was adjusted to pH 7.65 and the solution was filtered through a 0.2 µm Sartorius Sartolab-V150 disposable filter unit. Media was stored at 4°C. Liquid DME/F-12 1:1 mixture (glutamine-free) was prepared as above except that 24 ml 7.5 % (w/v) NaHCO₃ solution was used to give the same final concentration (3 g/l).

2.2.15 THE CELL CULTURE CONDITIONS

HEK 293 cells were grown in DME/F-12 medium in an incubator at 37°C and at a CO₂ content of 5 %. After two to three days, cells were subcultured as follows: media was removed and the cells were washed with 6 ml prewarmed HBSS and incubated in 1ml porcine trypsin 0.5 g/l and EDTA 0.2 g/l of HBSS (1 x trypsin-EDTA), at 37°C in the incubator. Enzyme dissociation was stopped by the addition of DME/F12 medium (10 ml). Finally, cells were diluted 5 x in DME/F-12 medium and grown in 10-15 ml of DME/F-12 medium.

2.2.16 LIQUID NITROGEN STORAGE OF HEK 293 CELLS

For long term storage, cells were dissociated using trypsin-EDTA (2.2.15) and pelleted at 200 x g for 5 min at 4°C. The cells were resuspended in 4.8 ml DME/F-12 medium and mixed with 0.6 ml FCS and 0.6 ml dimethyl sulphoxide (DMSO, ≥ 99.9 %), immediately divided into three freezing vials, placed in a polystyrene box and stored at -80°C for 24 h before transfer to liquid nitrogen.

When required, the cells were quickly thawed in a water bath at 37°C, pelleted by centrifugation at 200 x g for 5 min and resuspended in DME/F-12 (15 ml). The cells were placed in an incubator and cultured as described above.

2.2.17 Ca²⁺ PHOSPHATE-MEDIATED TRANSFECTION OF HEK 293 CELLS

HEK 293 cells were transfected using the calcium phosphate precipitation method as described in Gorman *et al.*, (1990).

The following stock solutions were prepared:

- (a) 2 x N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffered saline, 50 mM HEPES, pH 7.12, 280 mM NaCl, 1.1 mM Na₂HPO₄ (2 x HBS).
- (b) 2.5 M CaCl₂.
- (c) TE buffer, pH 8.0, diluted 1:10 in water.
- (d) Phosphate Buffered Saline, 4 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 107 mM KCl, (PBS)
- (e) 15 % (v/v) glycerol in PBS buffer.

HEK 293 cells were cultured in DME/F-12 medium. At 24 h prior to transfection, cells were plated to a density of 4 x 10⁶ per 250 ml flask (2.2.19). The media was changed 3 h prior to transfection with 10 ml/flask fresh growth media and cells placed in an incubator which was set for the media pH to be pH 7.3-7.4 at the moment of transfection. Two tubes were prepared. In tube A, the following were mixed: 440 µl diluted TE buffer, 10 µl appropriate DNA (1 µg/µl) and 50 µl 2.5 M CaCl₂. In tube B, 500 µl 2 x HBS was added. The content of tube A was added dropwise and slowly to tube B and the mixture was pipetted up and down several times. The precipitate was added directly to the cells in culture and the contents were swirled over the cells for even distribution. Cells were incubated for 3 h in a CO₂ incubator at 37°C. Media was aspirated from the cells and 1.5 ml 15 % (v/v) glycerol in PBS was incubated with cells

for 30 sec. Glycerol was removed by pipette, cells washed with media and incubated in 10 ml of fresh media in the absence or presence of NMDA receptor ligands. Cells were harvested up to 62 h post-transfection and processed as described in Chazot *et al.*, (1992). When HEK 293 cells were transfected to be used for single-channel recordings, immediately upon transfection cells were subcultured and transferred onto the poly-L-lysine coated CELLocate coverslips and grown in the presence of AP5 in the culture medium.

Plasmids used for cell transfection were: the pSV- β -galactosidase (2.1.4); the pCISNMDAR1-1a alone (2.3.1); the pCISNMDAR2A alone (2.3.2); the pCISNMDAR1-1a /pCISNMDAR2A together with DNA ratios as described in the results section.

The ligands used in the cell culture medium post-transfection were: AP5, 200 μ M; DKA, 200 μ M; Mg²⁺, 100 μ M, and CNQX, 200 μ M. The ligands were added two times, 0 h post-transfection and 6 h post-transfection.

2.2.18 DETERMINATION OF TRANSFECTION EFFICIENCY OF HEK 293 CELLS

Transfection efficiency was determined using the control vector pSV- β -galactosidase, with transfected cells identified by the use of x-gal as described in Lim and Chae (1989). The plasmid pSV- β -galactosidase is a control vector designed to express the enzyme β -galactosidase in mammalian cells. This enzyme is able to use the pseudo-substrate, x-gal, producing a coloured product. In this vector, the lacZ gene which encodes β -galactosidase is under the control of the SV40 early promoter and enhancer.

The following stock solutions were prepared:

- (a) 0.1 M sodium phosphate buffer, 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, pH 7.0.
- (b) x-gal solution, 10 mM sodium phosphate, 1 mM MgCl₂, 150 mM NaCl, 3.3 mM K₄[Fe(CN)₆]·3H₂O, 3.3 mM K₃[Fe(CN)₆], x-gal 0.2 % (w/v).

(c) glutaraldehyde solution, 100 mM sodium phosphate, 1 mM MgCl₂ and glutaraldehyde 1 % (v/v).

The HEK 293 cells were transfected with 10 µg pSV-β-galactosidase. Post-transfection (44 h), HEK 293 cells were washed five times with PBS buffer and fixed in a glutaraldehyde solution for 15 min at 4°C. Cells were then incubated in x-gal solution at 37°C for 1-2 h. Following incubation, the x-gal solution was removed and 70 % (v/v) glycerol was added to the dish. Transfected (blue) cells were identified under a light microscope (magnification, x 200). Total cell number and number of transfected cells were determined in three fields of view for each flask.

2.2.19 VIABLE CELL NUMBER DETERMINATION

Viable cell number was determined by trypan blue exclusion. HEK 293 cells were harvested using trypsin-EDTA (2.2.13) and centrifuged at 300 x g for 10 min at 4°C. The pellet was resuspended in 10 ml HBSS. The cell suspension (0.2 ml), was mixed with 0.3 ml HBSS and 0.5 ml 0.4 % (w/v) trypan blue solution and incubated at room temperature for 10 min. Drops of trypan blue-cell suspension were transferred using a pasteur pipette to both chambers of the haemocytometer and covered by a cover slip. Viable and non-viable (blue) cells were counted in ten fields from three independent samples for each culture flask. Cell viability and cell death were calculated by using the following formulae:

$$\text{CELL VIABILITY (\%)} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

$$\text{CELL DEATH (\%)} = \frac{\text{total non-viable cells (stained)}}{\text{total cells (stained and unstained)}} \times 100$$

2.2.20 CytoTox 96™ NON-RADIOACTIVE CYTOTOXICITY ASSAY

The CytoTox 96™ assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. The original protocol supplied by the manufacturer was adapted for the measuring of LDH released from transfected HEK 293 cells. The HEK 293 cells were transfected as described in 2.2.19 and 20 h following glycerol shock, samples of medium from experimental flasks were collected (1 ml). All samples were centrifuged at 4°C for 5 min and supernatants were transferred to fresh microfuge tubes. The samples were diluted where necessary and 50 µl were transferred in triplicate to 96-well plates. In all sample wells 50 µl of reconstituted substrate mix in assay buffer was added and plate was covered with the aluminium foil. The assay plate was incubated for 30 min at room temperature. The control flasks were used to measure spontaneous LDH release, background LDH of medium and maximum LDH release from the cells of the same density as the experimental flasks. A flask with HEK 293 cells for determination of maximum LDH release was incubated at -70°C for 30 min and at 37°C for 15 min to achieve complete lysis of the cells and the sample of 1 ml was taken. Control samples were assayed together with experimental samples as described above. Stop solution (50 µl) was added to each sample well and control wells at the end of 30 min incubation. The absorbance was recorded at $\lambda = 490$ nm using a Dynatech minireader. The average of absorbance from the culture medium background was subtracted from all absorbance values of experimental samples, spontaneous LDH release and maximum LDH release. The percent of cytotoxicity was calculated using the corrected values and the following formula.

$$\% \text{ CYTOTOXICITY} = \frac{\text{experimental} - \text{spontaneous release}}{\text{maximum} - \text{spontaneous release}} \times 100$$

2.3 RESULTS

Transient expression of genes in mammalian cells allows production of the proteins for structural and biochemical studies. Many methods of gene transfer have been developed using a suitable expression vector, host cell line and the transfection method for introducing DNA into cells. The host-vector system that includes the HEK 293 cell line (2.1.2) and the CMV promoter/enhancer based vector, particularly pCIS (2.1.4), has been used successfully for the expression of membrane-bound neurotransmitter receptors, such as e.g. GABA_A receptors (Pritchett *et al.*, 1988), glycine receptors (Pribilla *et al.*, 1992) and non-NMDA glutamate receptors (Werner *et al.*, 1991). Receptors expressed using this system had properties similar to receptors in the native system. We have chosen this host-vector system as a model system for the study of the molecular properties of the NMDA receptor.

2.3.1 SUBCLONING OF THE NMDAR1-1a cDNA INTO THE pCIS EXPRESSION VECTOR

The cDNA encoding the full length rat NMDAR1-1a subunit was excised from pN60 plasmid (Figure 2.3.1, Moriyoshi *et al.*, 1991) and cloned directionally into the *Sma I* / *Xba I* cloning sites of the pCIS mammalian expression vector (Figure 2.3.2), generating the recombinant pCISNMDAR1-1a (Figure 2.3.4). The subcloning strategy is summarized in Figure 2.3.5. Although different subcloning strategies could be chosen, we chose the strategy which resulted in reduction of the 5' and 3' untranslated regions to a minimum. It has been observed by other research groups that reduction of 5' untranslated regions to a minimum results in a better translation efficiency (Williams, 1993).

The full length rat NMDAR1-1a cDNA within the pN60, consists of a large open-reading frame encoding 938 amino acids and both 5' and 3' non-

coding regions of 265 and 868 bp respectively (Figure 2.3.1). The restriction enzyme *Nru I* was used to cut the clone at position -55 bp from the start codon producing blunt-end termini. The restriction enzyme *Nhe I* was used to cut the clone at a 3' site reducing the 3' untranslated region to 318 bp and generating protruding termini compatible to termini produced by *Xba I*. As a result of restriction enzyme digestion, a fragment of 3.7 kb was generated carrying a 5' blunt-end and a 3' cohesive end. In the same digestion reaction with *Nru I*, *Sca I* was used to cut the Bluescript part of the pN60 into two fragments of 2.1 kb and 1.4 kb respectively, thus reducing the possibility for false recombinants.

The plasmid pCIS was linearised using the *Sma I* and *Xba I* restriction enzymes. The resulting termini were compatible to 3.7 kb fragment of NMDAR1-1a cDNA. The DNA fragments, generated by restriction enzyme digestion were separated on LMP agarose gel and cut out as described in 2.2.13. The ligation reaction was set with the melted gel slices as described in 2.2.9. INV1 α competent cells were transformed with 2 μ l (30 ng of DNAs) the melted ligation reaction as described in 2.2.4. The bacterial colonies carrying recombinant DNA were grown over-night and the small-scale plasmid preparations were carried out (2.2.5.1). The plasmid DNAs (2 μ l) were loaded in parallel gel lanes together with the plasmid DNA fragments generated by restriction enzymes. Figure 2.3.3, lanes 1-3, shows the non-recombinant pCIS and the recombinant pCISNMDAR1-1a, lanes 4 and 5. The correct size of pCISNMDAR1-1a, 8.4 kb, was verified by restriction enzyme digestion. The results are shown in Figure 2.3.3. The resultant DNA fragments, 1.1, 2.6 and 4.7 kb, for the *Eco RI*, *Hind III* digestion, corresponded to the correct size of the recombinant, Figure 2.3.4.

2.3.2 SUBCLONING OF THE NMDAR2A cDNA INTO THE pCIS EXPRESSION VECTOR

The full length NMDAR2A clone within the pBKSA ϵ 1 consists of the open-reading frame encoding 1445 amino acids (Figure 2.3.6). It has also a 3' untranslated region of ~ 1.0 kb making together a 5.3 kb clone. To subclone the NMDAR2A cDNA from the pBKSA ϵ 1 (Meguro *et al.*, 1992, Figure 2.3.6) into the pCIS (Figure 2.3.2) firstly, a single step subcloning procedure was tried. This involved excision of the NMDAR2A cDNA from pBKSA ϵ 1 with *Xba I* and non-directional ligation with the pCIS. The pCIS was also linearised with *Xba I* and dephosphorylated as described (2.2.8). As this approach did not give a recombinant a two step subcloning procedure was carried out to yield the recombinant pCISNMDAR2A. The two step subcloning strategy is summarized in Figure 2.3.7.

Part of the NMDAR2A cDNA encoding the first 895 amino acids was cut out from the pBKSA ϵ 1 using *Xba I* and internal *Hind III* site (Figure 2.3.6). The resulting 2.7 kb fragment was subcloned into *Xba I / Hind III* polycloning site of pCIS to yield pCISNMDAR2Ashort (Figure 2.3.8, lanes 2-4 and Figure 2.3.9). As two different fragments were generated after *Xba I / Hind III* digestion of pBKSA ϵ 1 and both fragments were the same size of 2.7 kb, two different ligation products were also produced. The first part of the NMDAR2A clone, 1-2700 bp fragment, did not contain a *Nru I* site. The second part of the NMDAR2A clone, 2700-5400 bp, contained a single *Nru I* site (Figure 2.3.6), thus allowing us to distinguish the two different ligation products by digestion with *Nru I*. The results are shown in Figure 2.3.8. Lanes 5 and 7 show ligation product where the first cDNA part, 1-2700 bp, was subcloned in the correct orientation to yield the pCISNMDAR2Ashort. In lane 6, a second type of ligation product is represented which contains a single *Nru I* site (cut DNA).

The purpose of subcloning the *Bam* *HI* / *Eco* *RI* fragment of the pBKSA ϵ 1 into the pBluescript II KS (Figure 2.3.10) was to generate a *Hind* *III* site at the 3' end of the NMDAR2A cDNA. Thus, this *Hind* *III* site allowed subcloning of the second part of the NMDAR2A clone, *Hind* *III* / *Hind* *III* fragment, Figure 2.3.11, into the single *Hind* *III* site of the pCISNMDAR2Ashort to yield the pCISNMDAR2A. Figure 2.3.8, lane 16, shows the recombinant pBLUKS2Ashort cDNA. Lanes 17 and 18, show restriction enzyme analysis with control enzymes *Hind* *III* and *Hind* *III*, *Eco* *RI* generating the right size fragments, 2.7 and 4.4 kb.

The pBLUKS2Ashort was cut with *Hind* *III* and fragments were separated on an agarose gel (2.2.12). A 2.6 kb *Hind* *III* / *Hind* *III* fragment (second part of the NMDAR2A clone) was excised from the agarose gel and purified by using QIAEX matrix (2.2.13). The recombinant pCISNMDAR2Ashort was linearised with *Hind* *III* and cohesive ends were dephosphorylated (2.2.8). In the ligation reaction, a DNA ratio, foreign DNA:plasmid 2:1 (molar ratio) was used. The ligation reaction was carried out in 20 μ l total volume as described in 2.2.9. The competent *E. coli* cells, INV1 α strain, were transformed with 3 μ l of ligation reaction (2.2.4) and small-scale plasmid preparations were analysed by flat bad gel electrophoresis. The results are shown in Figure 2.3.13. The recombinant pCISNMDAR2A DNAs are in lanes 13-17. As the 2.6 *Hind* *III* / *Hind* *III* insert was subcloned in both orientations, the correct orientation was determined by restriction enzyme digestion with *Eco* *RI*. The full length NMDAR2A cDNAs subcloned in the sense orientation are represented in lanes 8 and 10. The *Hind* *III* / *Hind* *III* insert subcloned in antisense orientation is represented in lane 9.

Thus, as a result of a two step subcloning procedure, the recombinant pCISNMDAR2A was constructed in the correct orientation (Figure 2.3.12).

2.3.3 TRANSIENT EXPRESSION OF THE pCISNMDAR1-1a RECOMBINANT IN MAMMALIAN CELLS

In order to obtain the maximal expression of NMDA receptors in mammalian cells, initial experiments were carried out to optimize the efficiency of the transfection procedure. Following transfection with the control vector pSV- β -galactosidase, the efficiency of transfection was determined by x-gal staining. Once successful transfection with the pSV- β -galactosidase had been achieved, the parameters in the transfection procedure were optimised. These parameters were the pH of the media and the cell number at the moment of transfection. Table 2.3.1 shows results obtained during optimisation of these important parameters. It can be seen that the percentage of transfected cells increased with pH. The optimal pH values could not be achieved by adjusting either the CO₂ level or the pH of prepared media alone. As Table 2.3.2 illustrates, changing the NaHCO₃ content from 1.2 g/l to 3 g/l stabilised the pH at the critical moment of transfection and that content was used in all subsequent transfection experiments. During initial experiments, different cell densities were tested. These results are summarised in Table 2.3.1. The number of cells refers to the number of cells per flask (250 ml), seeded 24 h prior to transfection. The most satisfactory results were accomplished by seeding 4-5 x 10⁶ cells per flask, which resulted in a cell density of 40 - 60 % at the moment of transfection. Also, different amounts of total DNA per flask were tested. Results obtained with 10 or 15 μ g per flask were not significantly different, but 20 μ g DNA per flask resulted in a lower transfection efficiency (results not shown). Following optimisation experiments, cells were transfected routinely with 10 μ g total DNA per flask. Using these established conditions for transfection, an efficiency of 20 \pm 2 % was routinely achieved. An example of determination of transfection efficiency is shown in Figure 2.3.14.

When the optimal transfection conditions were established, HEK 293 cells were transfected with the pCISNMDAR1-1a subunit alone. Expression of the NMDAR1-1a subunit was determined by both immunoblotting with anti-NMDAR1-1a antibodies and by [³H]MK801 specific binding activity. Thus [³H]MK801 bound specifically and to saturation to a single high affinity site of $K_D = 8.5 \pm 2.5$ nM (n=3) and $B_{max} = 113 \pm 37$ fmol [³H]MK801 binding sites/mg protein (n=10) in homogenates of transfected HEK 293 cells. Figure 2.3.15 shows an immunoblot with HEK 293 cell homogenates. Following transfection with pCISNMDAR1-1a, two immunoreactive species with M_r 117 000 and M_r 97 000 were detected when transfected HEK 293 cells were used as antigen. Both of these immunoreactive species were blocked by preincubation with NMDAR1-1a (929-938) peptide. When non-transfected cells were used, no immunoreactivity was detected in Western blots. N-Deglycosylation of the NMDAR1-1a polypeptide resulted in a single immunoreactive species with M_r 97 000.

CHO cells were also transfected with the pCISNMDAR1-1a alone. Expression of the NMDAR1-1a subunit was demonstrated by immunoblotting. When the same amount of protein from transfected HEK 293 and CHO cells were analysed by immunoblotting, significantly lower immunoreactivity was detected on proteins from transfected CHO cells (Figure 2.3.16). Transfection efficiency with the control vector pSV- β -galactosidase for CHO cells was 5-10 %, n = 2.

2.3.4 TRANSIENT EXPRESSION OF THE pCISNMDAR2A RECOMBINANT IN MAMMALIAN CELLS

Using the established transfection conditions as above, the pCISNMDAR2A recombinant was transfected alone in HEK 293 cells. Expression of the NMDAR2A subunit was demonstrated by immunoblotting with

anti-NMDAR2A antibodies but no [³H]MK801 specific binding activity was detected in transfected HEK 293 cell homogenates. Figure 2.3.17 shows an immunoblot with HEK 293 cells transfected with the pCISNMDAR2A recombinant. A single immunoreactive species of M_r 180 000 was detected. Following N-deglycosylation of the NMDAR2A polypeptide, a single immunoreactive species of M_r 165 000 was detected.

2.3.5 TRANSIENT CO-EXPRESSION OF THE pCISNMDAR1-1a AND THE pCISNMDAR2A RECOMBINANTS

The co-transfection was done essentially as described in 2.2.17, maintaining the total amount of plasmid DNA added per flask at 10 µg. It was observed in initial experiments that after 16 h post-transfection, HEK cells changed their morphology and became detached from culture flask. Western blots of these co-transfected cells gave no immunological signals. In the same membranes from co-transfected cells, no [³H]MK801 specific binding activity was detected. When the co-transfected HEK 293 cells were grown in the presence of the NMDA receptor antagonist AP5, cells remained viable and both the NMDAR1-1a and NMDAR2A subunits were detected immunologically for up to 62 h (see 2.3.5.1.1 and Figure 2.3.19). Specific [³H]MK801 binding activity in cell homogenates was also detected with up to a 10-fold increase in binding activity with the respect to HEK 293 cells expressing the NMDAR1-1a subunit alone (Figure 2.3.18).

2.3.5.1 Optimisation of conditions for the co-expression of NMDAR1-1a /NMDAR2A heteromeric receptors in HEK 293 cells, grown in the presence of AP5

To optimise conditions for the expression of both NMDAR1-1a and NMDAR2A subunits with maximal functional responses, further experiments were carried out. These experiments included a time-course for the expression of subunits post-transfection and optimisation of the DNA ratios used for transfection.

2.3.5.1.1 *Time-dependence for the expression of NMDAR1-1a/NMDAR2A heteromeric receptors*

Because during initial coexpression experiments a cell toxicity problem was observed, we have further investigated transfection of the HEK 293 cells to maximise expression of heteromeric NMDA receptors. The time-dependent appearance of immunoreactivity was firstly investigated (Figure 2.3.19). As it can be seen in co-transfection experiments, the appearance of the immunoreactive species was identical for both subunits. The immunoblot showed that immunoreactivity of both NMDAR1-1a and NMDAR2A subunits could be detected at 3 h post-transfection (note that this is not seen for exposure time shown in Figure 2.3.19). It reached significant levels after 16 h, with maximal immunoreactivity observed at 24 h. Approximately the same level of immunoreactivity could be observed for up to 62 h.

Specific [³H]MK801 binding activity followed the same time-dependent pattern, with a peak of binding sites at a time of 24 h which ^{is} stable for up to 62 h post-transfection (Figure 2.3.20). The cell division was attenuated at 24 h post-transfection, a time point which was concomitant with the appearance of the non-N-glycosylated polypeptide (not shown).

2.3.5.1.2 *The pCISNMDAR1-1a / pCISNMDAR2A DNA ratio used for cell transfection*

The cDNAs encoding NMDAR1-1a and NMDAR2A have significantly different sizes, 2.8 kb and 4.3 kb respectively which may result in different expression levels. Susceptibility to mRNA degradation and protein stability could also be different for the different subunits. Because of these possible differences and the unknown stoichiometry of NMDA receptors, the DNA ratio of the pCISNMDAR1-1a:pCISNMDAR2A in transfection experiments was investigated. Earlier studies had showed that 10 µg total plasmid DNA was optimal for the transient expression of single NMDA receptor subunit (2.3.3). Therefore, in all subsequent experiments, the DNA ratio was varied maintaining the total amount of DNA at 10 µg. [³H]MK801 binding assays were performed on membranes prepared from transfected HEK 293 cells and the results are shown in Figure 2.3.21. The maximum expression was achieved with DNA ratios of 1:3 and 1:5 for pCISNMDAR1-1a:pCISNMDAR2A respectively. The intensity of immunoreactive bands on the immunoblot correlated well with the amount of DNA of each respective subunit for the different DNA ratios used (not shown).

In conclusion, the optimum conditions for co-expression of NMDAR1-1a/NMDAR2A receptors were a 1:3 DNA ratio, the presence of AP5 in the culture medium, with cell harvesting 24 h post-transfection. These conditions were used in all subsequent experiments.

When the optimal conditions were used, [³H]MK801 bound to cotransfected cell membranes with a single high affinity site with $K_D = 6.1 \pm 1.4$ nM and a $B_{max} = 1053 \pm 185$ fmol/mg protein (n = 10) (Cik *et al.*, 1993). The affinity of this site is comparable to that determined for the singly expressed NMDAR1-1a subunit and for NMDA receptors in an adult rat brain (Chazot *et al.*, 1992). The number of binding sites present in the co-transfection experiments showed an approximate 10-fold increase in comparison to the NMDAR1-1a

expressed alone. The pharmacological profile of [³H]MK801 specific binding activity to recombinant NMDA receptor was similar to the native receptor, but a difference in sensitivity to the divalent cations Mg²⁺ and Ca²⁺ was apparent (Table 3.3.3).

2.3.5.2 Single-channel properties of the NMDAR1-1a/NMDAR2A expressed in HEK 293 cells

Following transfection of HEK 293 cells with the NMDAR1-1a/NMDAR2A subunit combination, cells were transferred to cover slips as described in 2.2.17 and grown in the presence of AP5 in the culture medium. Single-channel behaviour was recorded 20 h post-transfection from outside-out membrane patches (Stern *et al.*, 1994). Figure 2.3.22 shows a typical example of a recording from an outside-out patch obtained from a transfected HEK 293 cell. Single-channels showed a main conductance level of 51.4 ± 2.4 pS and a subconductance of 38.1 ± 2.1 pS. Distribution of shut times was fitted with 5 exponential components and distribution of all apparent open times was fitted with two exponential components.

2.3.5.3 Prevention of cell death by pharmacological manipulation

Although the co-transfected HEK 293 cells were grown in the presence of AP5 in the culture medium post-transfection, some cell death was still visually apparent compared to non-transfected or singly transfected HEK 293 cells. It has been described that a number of classes of pharmacologically different compounds antagonise the native NMDA receptor (Watkins *et al.*, 1990). The effect of some of these compounds on the survival of recombinant NMDAR1-1a/R2A receptors post-transfection was investigated. The co-transfected HEK 293 cells were grown in the presence of one, or a combination of two compounds

and their effect on the prevention of cell death was monitored by trypan blue exclusion or by CytoTox 96TM, non-radioactive cytotoxicity assay, as described in 2.2.19-20. The results are summarized in Table 2.3.3. The singly transfected HEK 293 cells, NMDAR1-1a or NMDAR2A had the same number of non-viable cells compared to non-transfected cells. When NMDAR1-1a and R2A subunits were co-transfected, cell death occurred. The presence of saturating concentrations of AP5 and the antagonist of the strychnine-insensitive glycine site, DKA, gave optimal protection against cell death with no significant difference between the percentage of dead cells in control and test samples. It was interesting that Mg²⁺ and even DKA alone both partially protected against cell death. The integrity of the NMDAR1-1a and NMDAR2A subunits was maintained, as determined by immunoblotting under all these protection conditions. The non-NMDA receptor antagonist, CNQX, had no effect on cell death.

The percentage cell death obtained with the co-transfection experiment of wild-type NMDAR1-1a/NMDAR2A subunits was similar to the transfection efficiency. To test if all transfected cells died, the pSV- β -galactosidase vector (2.1.4) was co-transfected together with the wild-type NMDAR1-1a/NMDAR2A subunits. The co-transfected cells were subsequently grown in the presence and absence of AP5 and DKA. The percentage cell death was assayed 20 h following co-transfection by CytoTox 96TM assay. In the same experimental flasks, the efficiency of transfection was estimated 44 h post-transfection by trypan blue exclusion as described in 2.2.19-20 (Table 2.3.4 and Figure 2.3.23). When co-transfected HEK 293 cells were grown in the absence of AP5 and DKA, no x-gal stained cells were detected and cell death was observed (21.4 %). The presence of AP5 and DKA in cell medium post-transfection resulted in no cell death and x-gal stained cells were observed (~ 19 %).

2.3.6 EFFECT OF TUNICAMYCIN ON ASSEMBLY OF RECOMBINANT HETEROMERIC NMDA RECEPTORS

Initially, HEK 293 cells were transfected with pCISNMDAR1-1a / pCISNMDAR2A recombinants and grown under optimal conditions as described previously (2.3.4.1.2), in the presence of 2 µg/ml tunicamycin, a selective inhibitor of asparagine-linked oligosaccharide synthesis. The presence of tunicamycin resulted in appearance of morphologically aberrant, rounded cells. The cells were harvested after 16 h and assayed for [³H]MK801 specific binding activity. Tunicamycin treatment resulted in the complete loss of specific [³H]MK801 binding activity compared to control cells grown in parallel in the absence of antibiotic. The subunit-specific anti-NMDAR1-1a and anti-NMDAR2A antibodies were used to probe these membrane preparations by immunoblotting analysis. The complete lack of N-glycosylation of the NMDAR1-1a subunit and greatly reduced N-glycosylation of the NMDAR2A subunit was observed, with no apparent qualitative loss of immunoreactivity (not shown).

When cells were grown in a range of concentrations of tunicamycin (0.02-2 µg/ml media), increase in unglycosylated NMDAR1-1a and NMDAR2A subunit polypeptides were observed following immunoblotting analysis (Figure 2.3.24). Tunicamycin had also a dose-dependent effect on [³H]MK801 specific binding activity, with intermediate levels of [³H]MK801 binding activity detected in membrane preparations derived from cells grown in the presence of 0.02 µg tunicamycin/ml media and 0.1 µg/ml media (not shown). A complete inhibition of glycosylation of the NMDAR1-1a subunit polypeptide and loss of [³H]MK801 specific binding activity was observed in the presence of 0.5 µg tunicamycin/ml media. No significant reduction in immunoreactivity was observed following the treatment with tunicamycin (Chazot *et al.*, 1994b).

To investigate the reversibility of the effect of tunicamycin, transfected cells were grown in the presence of 2 μg tunicamycin/ml media for 24 h following transfection and a further 38 h in tunicamycin free media. It was previously shown that functional expression of the NMDAR1-1a / NMDAR2A heteromeric receptor was stable for at least 62 h post-transfection (2.3.4.1.1). The inhibition of [^3H]MK801 specific binding activity following treatment with tunicamycin was completely reversible. The cells were also observed to recover their morphology to the appearance of control cells grown in parallel without treatment, demonstrating that tunicamycin was not deleterious to the cells. Immunoblots demonstrated that the change of media 24 h after tunicamycin treatment resulted in partial reglycosylation of the NMDAR1-1a subunit which appeared sufficient to promote efficient assembly of the functional receptor (not shown). Saturation binding analysis was performed to investigate whether the apparent reduction in [^3H]MK801 binding sites following tunicamycin treatment was due to a change in affinity of the receptor or a reduction in the total number of receptor sites. Transfected cells expressing heteromeric NMDA receptors were treated with 0.02 $\mu\text{g}/\text{ml}$ and 0.1 $\mu\text{g}/\text{ml}$ tunicamycin in media and their homogenates as well as homogenates from cells in which tunicamycin was removed after 24 h treatment were used for saturation binding analysis (Chazot *et al.*, 1993). The apparent loss of [^3H]MK801 binding sites was due exclusively to a progressive reduction in the affinity for [^3H]MK801, with no significant effect on the number of binding sites (Table 2.3.5). Following removal of the tunicamycin from media, after 24 h treatment, the K_D of [^3H]MK801 for the recombinant receptor returned to the control level.

number of cells	pH	% of stained cells	n
10 x 10 ⁶	6.8	N.D.	1
10 x 10 ⁶	7.1	3	1
4 x 10 ⁶	7.0-7.1	0	4
10 x 10 ⁶	7.2	5	1
2.5 x 10 ⁶	7.2	10	1
5 x 10 ⁶	7.4	17	1

Table 2.3.1 **The effect of pH and cell number at the moment of transfection on the efficiency of transfection.**

HEK 293 cells were seeded at different concentrations 24 h prior to transfection with pSV- β -galactosidase. At the moment of transfection the pH was measured with a hand-held pH meter. Cell viability was assayed 48 h post-transfection by trypan blue exclusion as described in 2.2.19.

n = number of separate experiments

N.D. = not determined

number of cells	pH	% of stained cells	NaHCO ₃ g/l	n
4 x 10 ⁶	6.8-7.1	~1	1.2	5
4 x 10 ⁶	7.2-7.3	22 ± 2	3.0	2

Table 2.3.2 The effect of NaHCO₃ content on the pH of the medium at the moment of transfection and on the efficiency of transfection.

HEK 293 cells were seeded 24 h prior to transfection and grown in medium with two different contents of NaHCO₃. Cells were transfected with pSV-β-galactosidase and 48 h following transfection cell viability was assayed by trypan blue exclusion as described in 2.2.19.

n = number of experiments

Transfection sample	cell mortality (%) measured by trypan blue exclusion	cell mortality (%) measured by LDH release
untransfected cells	3.3 ± 1.8 (2)	N.D.
NMDAR1-1a	3.2 ± 0.9 (3)	0.0 ± 0.2 (2)
NMDAR2A	2.4 ± 0.1 (2)	0.2 ± 0.8 (2)
NMDAR1-1a/NMDAR2A	21.7 ± 3.0 (11)	24.5 ± 2.3 (7)
NMDAR1-1a/NMDAR2A 2 x AP5 (200 µM each)	8.3 ± 2.6 (5)***	9.1 ± 1.0 (5)***
NMDAR1-1a/NMDAR2A 2 x DKA (200 µM each)	12.8 ± 3.7 (4)***	N.D.
NMDAR1-1a/NMDAR2A 2 x Mg ²⁺ (10 mM each)	13.6 ± 3.7 (3)**	N.D.
NMDAR1-1a/NMDAR2A 2 x AP5 + 2 x DKA (200 µM each)	2.8 ± 1.6 (3)*	0.0 ± 0.5 (3)
NMDAR1-1a/NMDAR2A 2 x AP5 (200 µM each) + 2 x Mg ²⁺ (10 mM each)	5.7 ± 1.9 (4)	N.D.
NMDAR1-1a/NMDAR2A 2 x DKA (200 µM each) + 2 x Mg ²⁺ (10 mM each)	5.2 ± 1.8 (3)	N.D.
NMDAR1-1a/NMDAR2A 2 x CNQX (100 µM each)	21.8 ± 4.6 (3)	N.D.

Table 2.3.3 **The effect of NMDA receptor ligands on cell survival following co-transfection with pCISNMDAR1-1a and pCISNMDAR2A: comparison of trypan blue exclusion and LDH release.**

HEK 293 cells were transfected with pCISNMDAR1-1a and pCISNMDAR2A alone or in combination and cultured for 20 h in the presence or absence of different pharmacological agents. Cell viability was determined by trypan blue exclusion and CytoTox 96 assay as in 2.2.19-20 for (n) separate experiments. Results were analysed using a two-tailed Student's t-test. In comparison to cells grown in the absence of AP5, the degree of significance was **P < 0.0002, ***P < 0.0001, In comparison to cells grown in the presence of AP5, the degree of significance was *P < 0.02. Values are the means ± S.D for (n) separate experiments. N.D. = not determined

Transfection sample	LDH release (%)		x-gal staining (%)	
	unprotected	protected AP5, DKA	unprotected	protected AP5, DKA
NMDAR1-1a/NMDAR2A/pSV- β -galactosidase	21.4 (1)	0.04 (1)	< 0.5 (1)	~ 19.0 (1)

Table 2.3.4 Comparison of LDH release and x-gal staining in the same experimental flask following cotransfection of pSV- β -galactosidase, pCISNMDAR1-1a and pCISNMDAR2A.

HEK 293 cells were co-transfected with the pSV- β -galactosidase, the pCISNMDAR1-1a, the pCISNMDAR2A and grown in the presence or absence of AP5 and DKA (200 μ M each). Cell viability was determined by trypan blue exclusion and the CytoTox 96TM assay in the same experimental flasks as described in 2.2.19-20.

Sample (n)	K _D (nM)
Control (3)	6.1 ± 1.4
0.02 µg/ml Tunicamycin (3)	10.8 ± 5.1
0.1 µg/ml Tunicamycin (3)	26.8 ± 5.8
2 µg/ml Tunicamycin for 24 h, following the tunicamycin-free medium for 48 h (4)	6.6 ± 1.6

Table 2.3.5 The effect of tunicamycin in culture medium post-transfection on properties of heteromeric NMDAR1-1a/NMDAR2A receptors

HEK 293 cells were transfected with both pCISNMDAR1-1a and pCISNMDAR2A and cultured for 24 h in the presence or absence of tunicamycin in the cell culture medium. To reverse the effect of tunicamycin, transfected HEK 293 cells were grown for 24 h in the presence of tunicamycin, medium was changed and cells grown for a further 48 h in tunicamycin-free medium. Cell homogenates were prepared and saturation binding isotherms were determined for [³H]MK801. Transfected cells were grown in the presence of AP5 (200 µM). Values are the means ± S.D for (n) separate experiments.

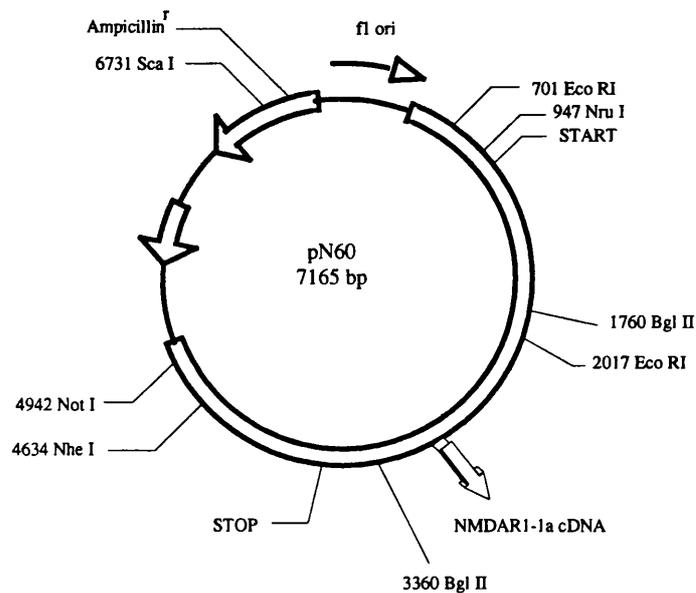


Figure 2.3.1 Restriction map of the plasmid pN60

The pN60 is an NMDAR1-1a cDNA containing pBluescript (Moriyoshi *et al.*, 1991). It was used as a donor of an NMDAR1-1a cDNA in a subcloning strategy generating the recombinant pCISNMDAR1-1a (2.3.1).

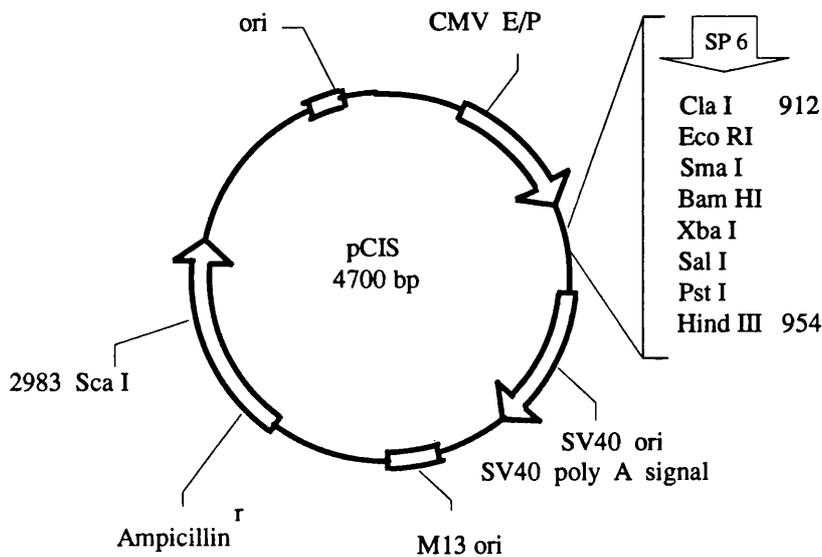


Figure 2.3.2 Restriction map of the plasmid pCIS

The pCIS is a mammalian expression vector that uses the CMV promoter/enhancer elements (2.1.4). The multiple cloning site has 7 unique cloning sites. ColE1 origin of replication (ori), permits replication in *E. coli* and the gene for ampicillin resistance allows selection of bacterial colonies harbouring plasmid DNA. Phage M13 origin of replication (M13 ori), permits ssDNA rescue (+ sense).

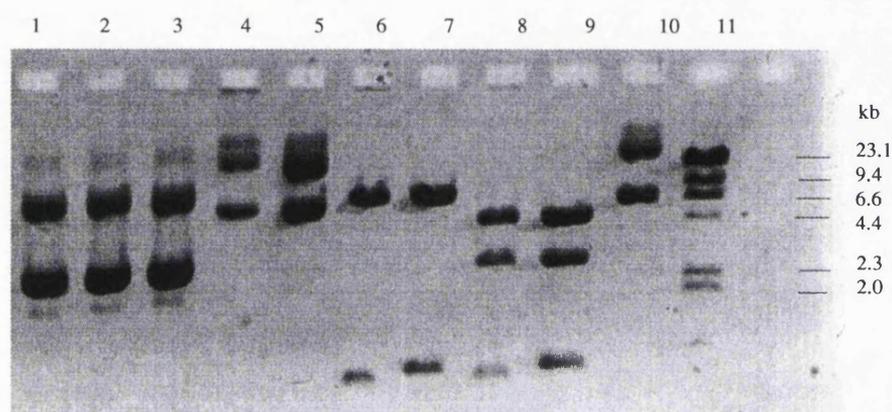


Figure 2.3.3 **Restriction enzyme analysis of the recombinant pCISNMDAR1-1a**

- Lanes 1-3 pCIS uncut
- Lanes 4-5, Recombinant pCISNMDAR1-1a uncut
- Lanes 6-7 Recombinant pCISNMDAR1-1a digested with *Eco RI*
- Lanes 8-9 Recombinant pCISNMDAR1-1a digested with *Eco RI* and *Hind III*
- Lane 10 Recombinant pCISNMDAR1-1a
- Lane 11 Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb)

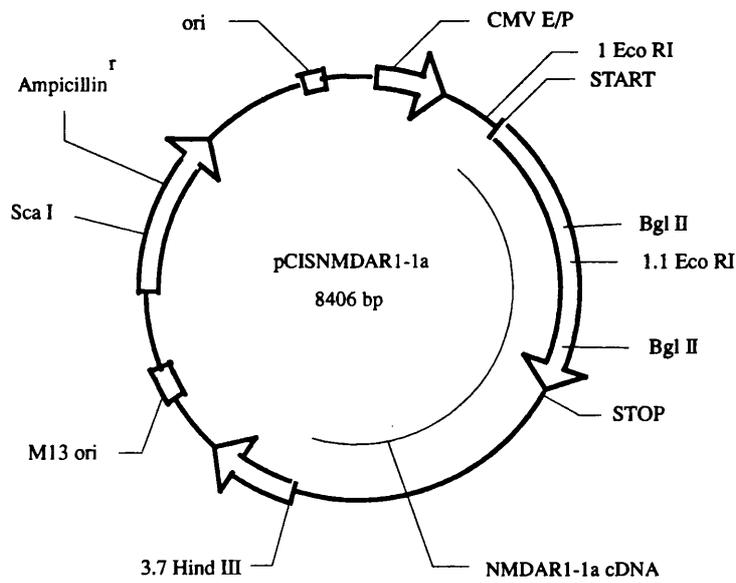


Figure 2.3.4 Restriction map of the recombinant pCISNMDAR1-1a

The recombinant pCISNMDAR1-1a was generated upon subcloning of the NMDAR1-1a subunit cDNA into the mammalian expression vector pCIS (2.3.1).

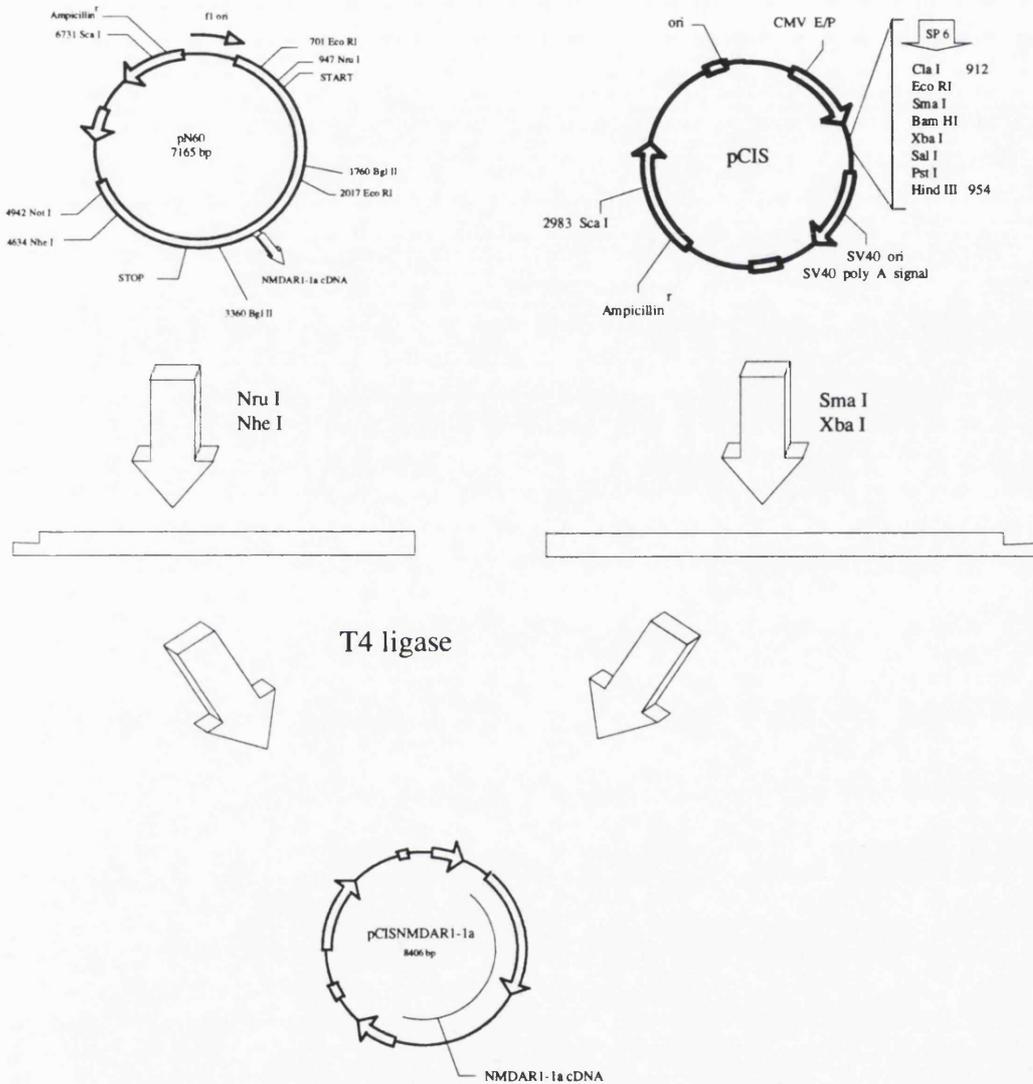


Figure 2.3.5 Subcloning strategy for recloning of the NMDAR1-1a into the mammalian expression vector pCIS.

The cDNA encoding the full length rat NMDAR1-1a subunit was excised from the pN60 plasmid and cloned directionally into the *Sma I / Xba I* cloning sites of the pCIS mammalian expression vector generating the recombinant pCISNMDAR1-1a.

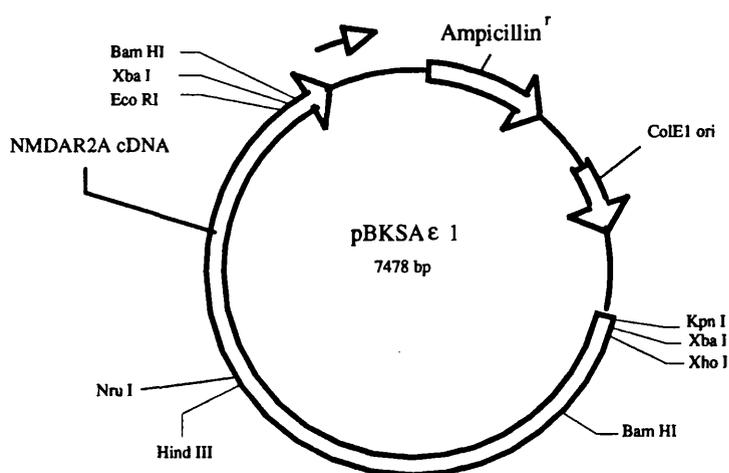


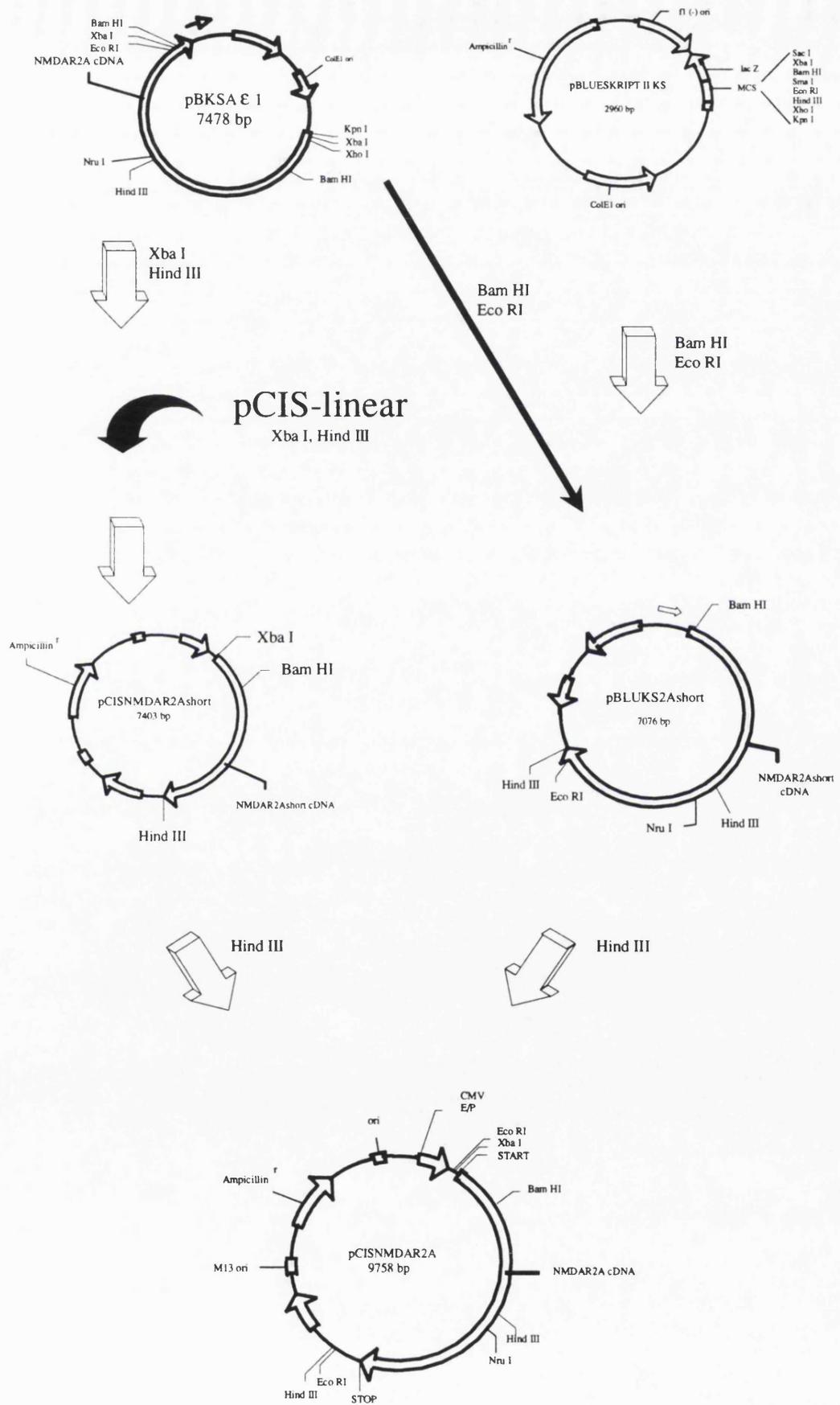
Figure 2.3.6 **Restriction map of the plasmid pBKSAε1**

pBKSAε1 is an ε1 (NMDAR2A) cDNA containing vector (Meguro *et al.*, 1992). It was used as a donor for NMDAR2A cDNA in a subcloning strategy aimed to generate the recombinant pCISNMDAR2A (2.3.2).

Figure 2.3.7 Subcloning strategy for recloning of the NMDAR2A into the mammalian expression vector pCIS.

The cDNA encoding the first 895 amino acids of the NMDAR2A was cut out from pBKSAε1 using *Xba I* and an internal *Hind III* site. The resulting 2.7 kb fragment was subcloned into the *Xba I* / *Hind III* polycloning site of pCIS to yield pCISNMDAR2Ashort. The purpose of subcloning the *Bam HI* / *Eco RI* fragment of the pBKSAε1 into the pBluescript II KS was to generate a *Hind III* site at the 3' end of the NMDAR2A cDNA. Thus, this *Hind III* site allowed the subcloning of the second part of the NMDAR2A clone, *Hind III* / *Hind III* fragment into the single *Hind III* site of the pCISNMDAR2Ashort to yield the pCISNMDAR2A.

Thus, as a result of a two step subcloning procedure, the recombinant pCISNMDAR2A was constructed in the correct orientation and used in transfection experiments.



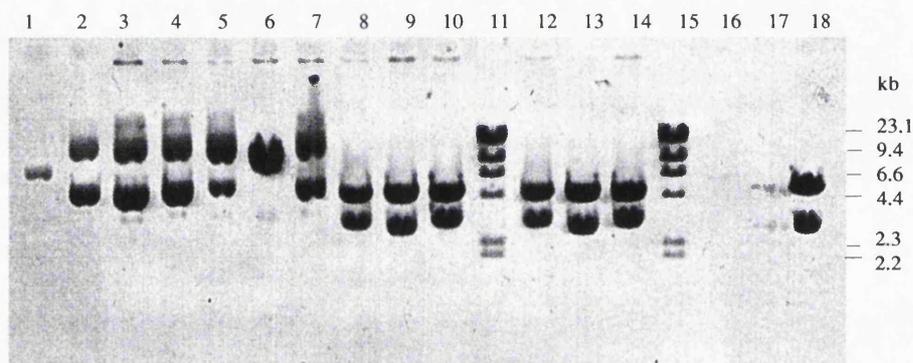


Figure 2.3.8 **Restriction enzyme analysis of the recombinants pCISNMDAR2Ashort and pBLUKS2Ashort**

- Lane 1 pCIS, uncut
- Lanes 2-4 pCISNMDAR2Ashort, uncut
- Lanes 5, 7 Recombinant pCISNMDAR2Ashort, there is no *Nru I* site, uncut
- Lane 6 Recombinant pCISNMDAR2Ashort, *Nru I* site is present, cut
- Lanes 8-10 Recombinant pCISNMDAR2Ashort digested with *Eco RI* and *Hind III*
- Lanes 11,15 Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb)
- Lanes 12-14 Recombinant pCISNMDAR2Ashort digested with *Xba I* and *Hind III*
- Lane 17 Recombinant pBLUKS2Ashort digested with *Hind III*
- Lane 18 Recombinant pBLUKS2Ashort digested with *Hind III* and *Eco RI*

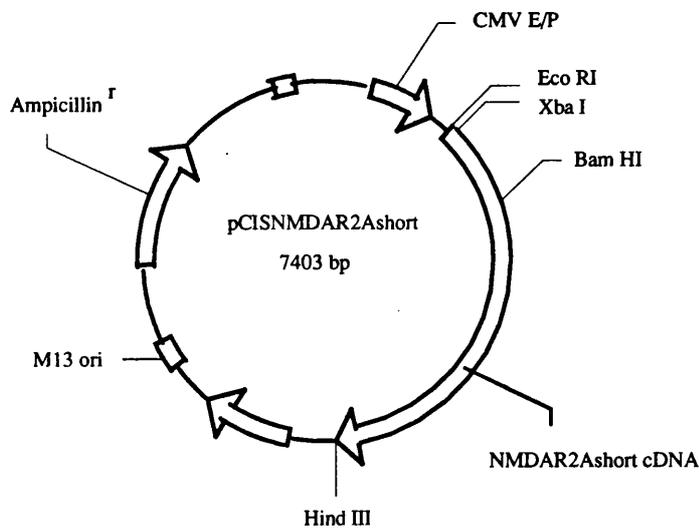


Figure 2.3.9 **Restriction map of the recombinant pCISNMDAR2Ashort**

Represents the recombinant pCISNMDAR2Ashort, containing the 2.7 kb cDNA fragment of NMDAR2A encoding 1-895 amino acids. It was used as an intermediate recombinant in a subcloning strategy aimed to produce pCISNMDAR2A (2.3.2.1).

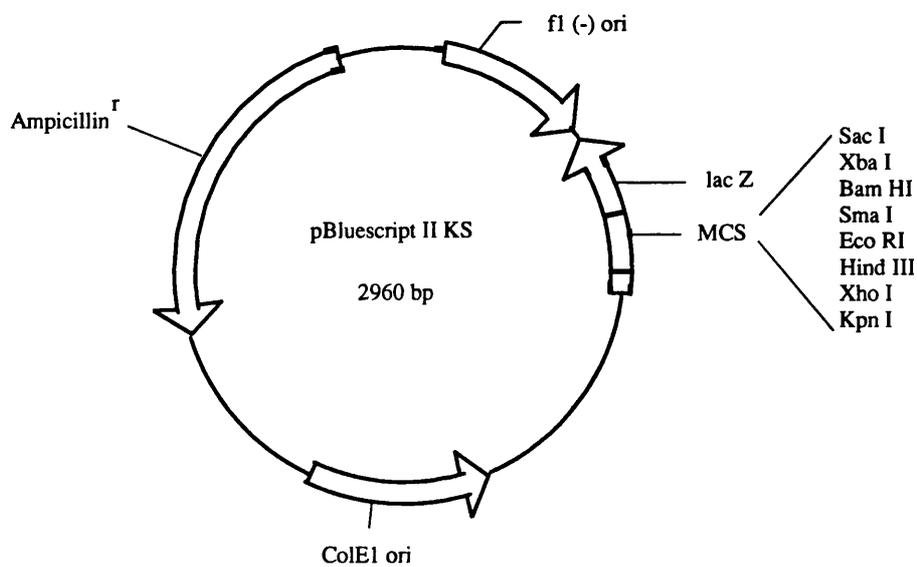


Figure 2.3.10 **Restriction map of the plasmid pBluescript II KS**

The pBluescript II KS (Stratagene Ltd. Cambridge, UK) is the vector used to engineer the *Hind III* site at the 3' end of NMDAR2A cDNA (2.3.1.1).

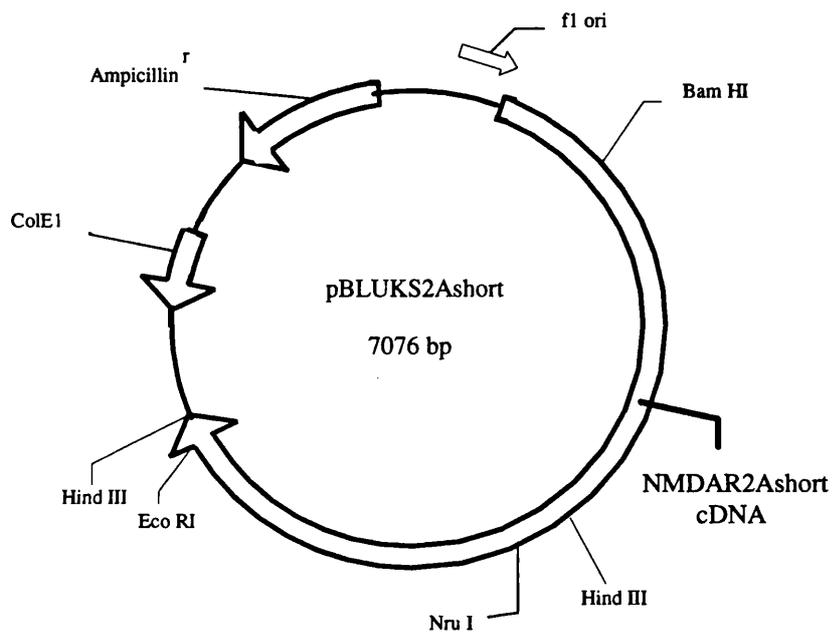


Figure 2.3.11 Restriction map of the recombinant pBLUKS2Ashort

This figure shows the recombinant pBLUKS2Ashort, obtained by subcloning of the 4.8 kb *Bam HI* / *Eco RI* fragment from pBKS Δ e1 into pBlueskript II KS (2.3.2.1). Note that at the 3' end of the 4.8 kb NMDAR2A cDNA there is *Hind III* site.

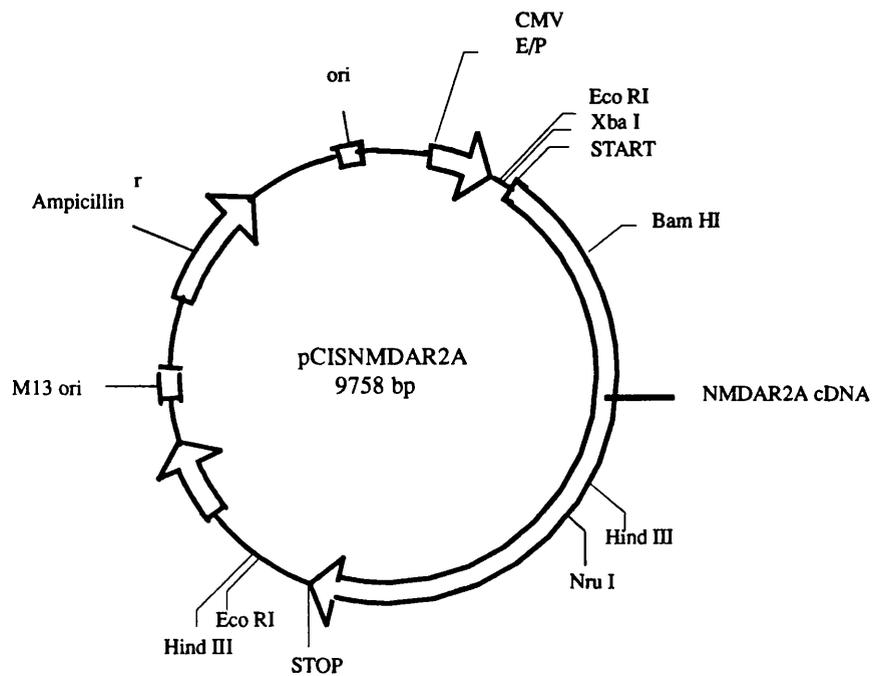


Figure 2.3.12 Restriction map of the recombinant pCISNMDAR2A

The figure shows the pCISNMDAR2A recombinant generated in the two step subcloning procedure (2.3.2.1-2.3.2.2).

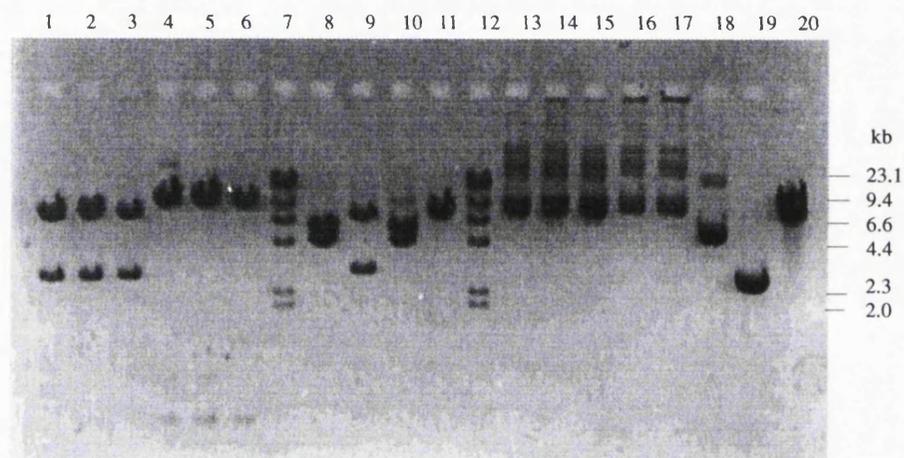


Figure 2.3.13 **Restriction enzyme analysis of the recombinant pCISNMDAR2A**

- Lanes 1-3 Recombinant pCISNMDAR2A digested with *Hind III*
- Lanes 4-6 Recombinant pCISNMDAR2A digested with *Bgl II*
- Lanes 7, 12 Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb)
- Lanes 8-10 Recombinant pCISNMDAR2A digested with *Eco RI*
- Lane 11 pCIS digested with *Eco RI*
- Lanes 13-17 Recombinant pCISNMDAR2A uncut
- Lane 18 pCIS uncut
- Lane 19 *Hind III* / *Hind III* fragment
- Lane 20 Recombinant pCISNMDAR2A short linearised with *Hind III*

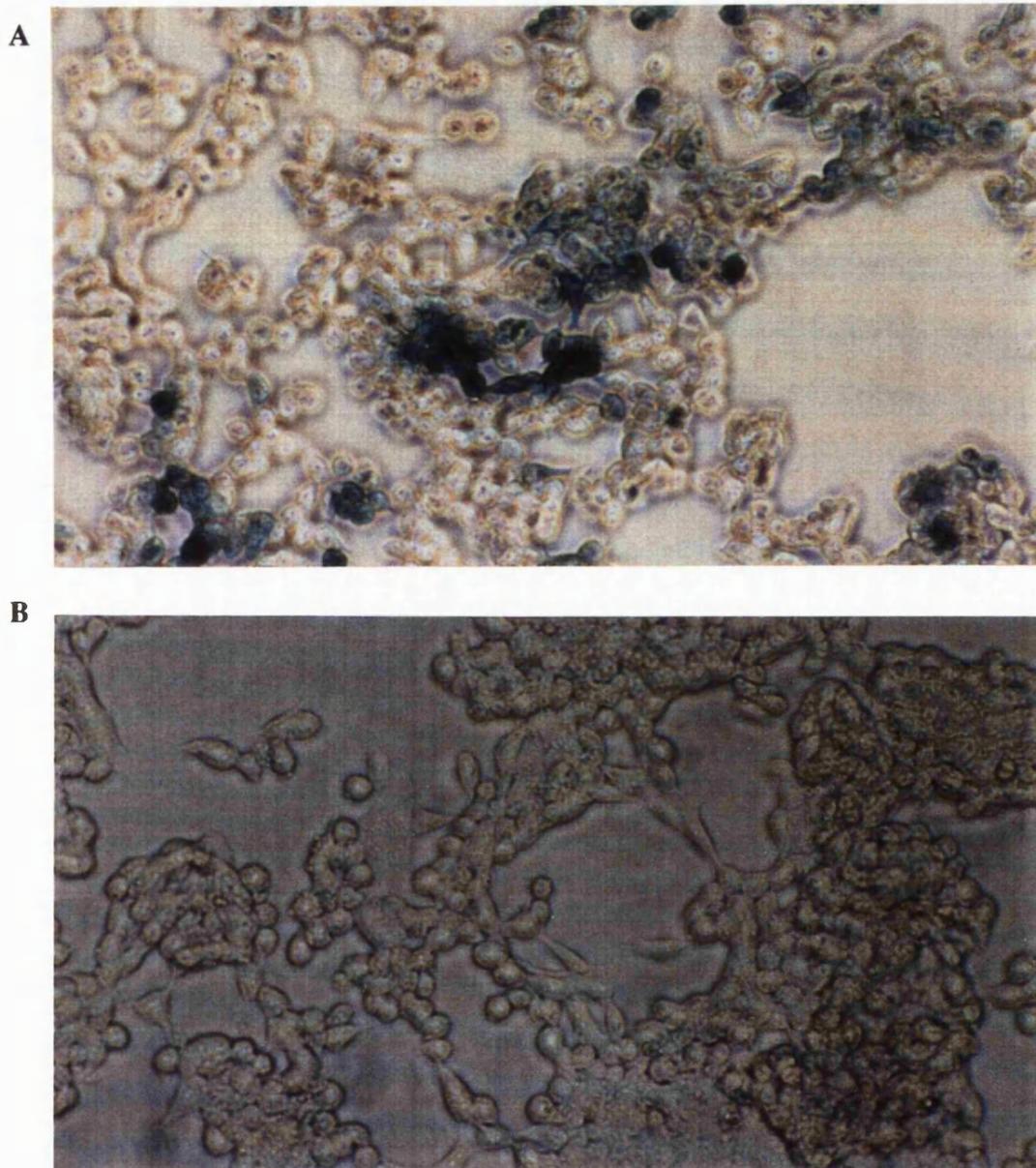


Figure 2.3.14 An example of determination of transfection efficiency with the vector pSV-β-galactosidase

HEK 293 cells were transfected with the pSV-β-galactosidase and stained with x-gal (A). Untransfected cells stained with x-gal (B). The photographs were taken on an inverted microscope and the film was developed by standard procedure.

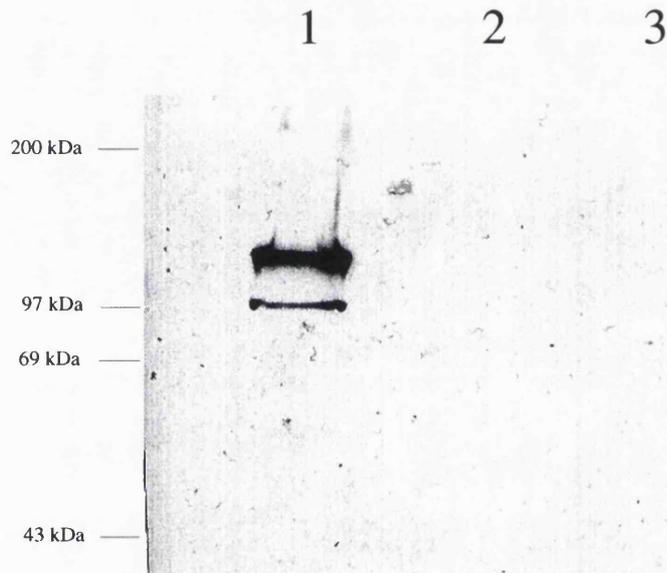


Figure 2.3.15 Immunoblot of HEK 293 cell homogenates probed with an NMDAR1-1a antibodies.

- Lane 1 Cells transfected with pCISNMDAR1-1a and blotted with anti-NMDAR1-1a antibodies
- Lane 2 Cells transfected with pCISNMDAR1-1a and blotted with anti-NMDAR1-1a antibodies; prior to immunoblotting antibodies were preincubated with NMDAR1-1a (929-938) peptide (100 µg/ml)
- Lane 3 Non-transfected HEK 293 cells probed with anti-NMDAR1-1a antibodies
- Affinity purified antibody concentration was (10 µg/ml)

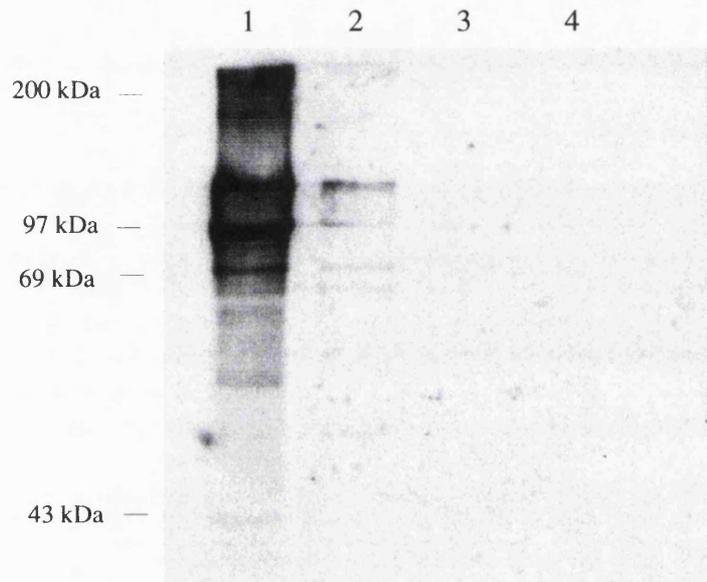


Figure 2.3.16 Comparison of immunoblots of HEK 293 and CHO cell homogenates probed with NMDAR1-1a antibodies

- Lane 1 HEK 293 cells transfected with pCISNMDAR1-1a and probed with anti-NMDAR1-1a antibodies
- Lane 2 CHO cells transfected with pCISNMDAR1-1a and probed with anti-NMDAR1-1a antibodies
- Lane 3,4 HEK 293 and CHO transfected cell homogenates, prior to immunoblotting antibodies were preincubated with NMDAR1-1a (928-939) peptide (100 $\mu\text{g/ml}$)
- Affinity purified antibody concentration was (10 $\mu\text{g/ml}$)

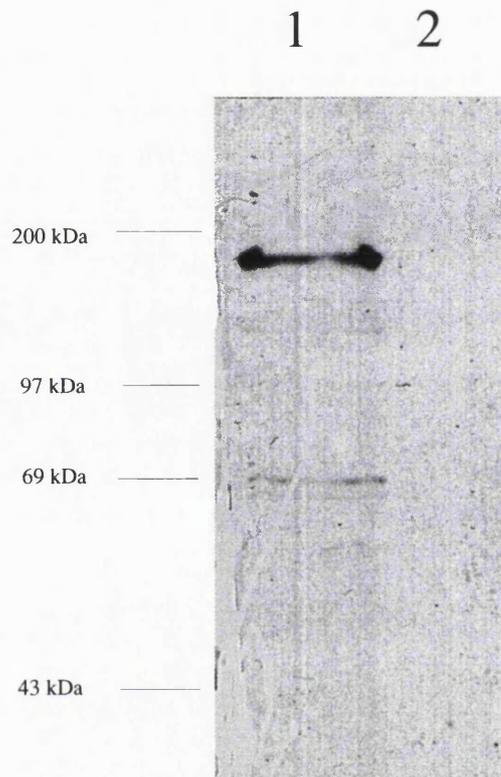


Figure 2.3.17 Immunoblot of HEK 293 cell homogenates probed with an NMDAR2A antibodies.

- Lane 1 HEK 293 cells transfected with pCISNMDAR2A and probed with anti-NMDAR2A (1435-1445) antibodies
- Lane 2 HEK 293 cells transfected with pCISNMDAR2A and probed with anti-NMDAR2A (1435-1445) antibodies; prior to immunoblotting antibodies were preincubated with NMDAR2A (1435-1445) peptide (100 µg/ml)
Affinity purified antibody concentration was (10 µg/ml)

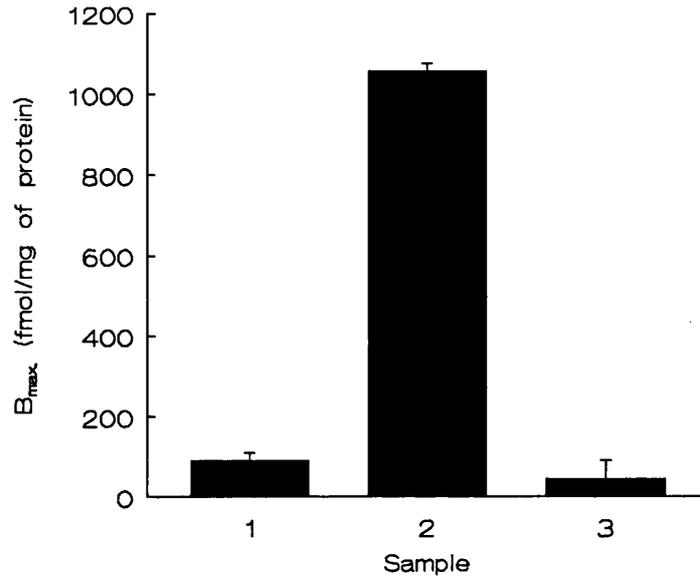


Figure 2.3.18 The effect of AP5 in the culture medium following HEK 293 cells transfection with pCISNMDAR1-1a and pCISNMDAR2A

HEK 293 cells were transfected with either pCISNMDAR1-1a alone or with both pCISNMDAR1-1a and pCISNMDAR2A and grown for 48 h in the presence or absence of AP5 (200 μ M). Values are the means \pm S.D. for three separate experiments.

- Lane 1 Cells transfected with pCISNMDAR1-1a and grown in the absence of AP5
- Lane 2 Cells transfected with pCISNMDAR1-1a and pCISNMDAR2A and grown in the presence of AP5
- Lane 3 Cells transfected with pCISNMDAR1-1a and pCISNMDAR2A and grown in the absence of AP5

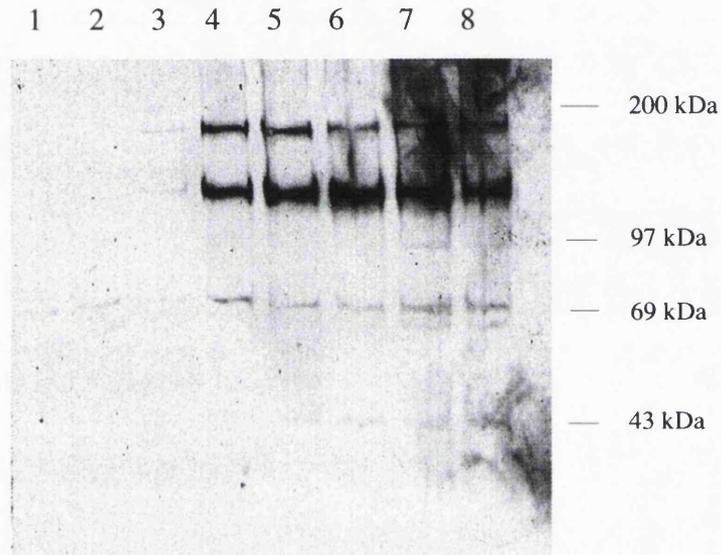


Figure 2.3.19 Time-dependence of the expression of NMDAR1-1a / NMDAR2A heteromeric receptors in HEK 293 cells

HEK 293 cells were transfected with both pCISNMDAR1-1a and pCISNMDAR2A, cultured in the presence of AP5 (200 μ M) and harvested at 0, 3, 6, 15, 24, 38, 48 and 62 h, lanes 1-8.

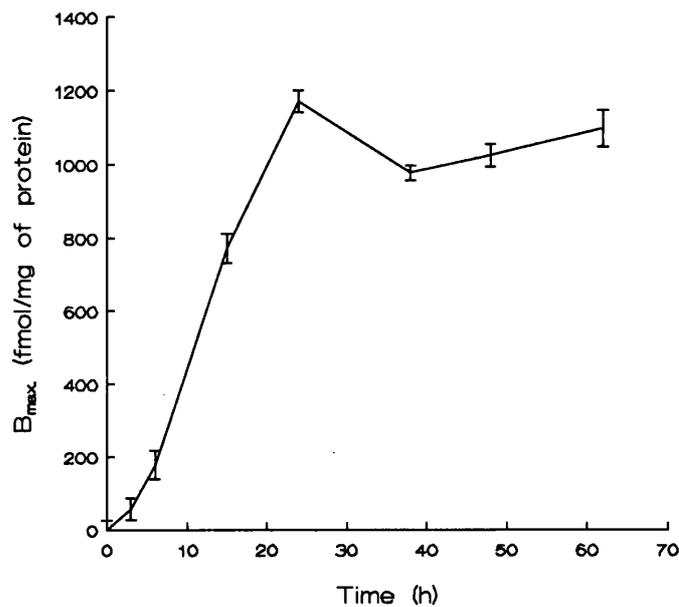


Figure 2.3.20 Time-dependence for the expression of $[^3H]MK801$ specific binding activity

HEK 293 cells were transfected with both pCISNMDAR1-1a and pCISNMDAR2A, cultured in the presence of AP5 (200 μM) and harvested at 0, 3, 6, 15, 24, 38, 48 and 62 h. Data are from a representative experiment which was performed three times with similar results.

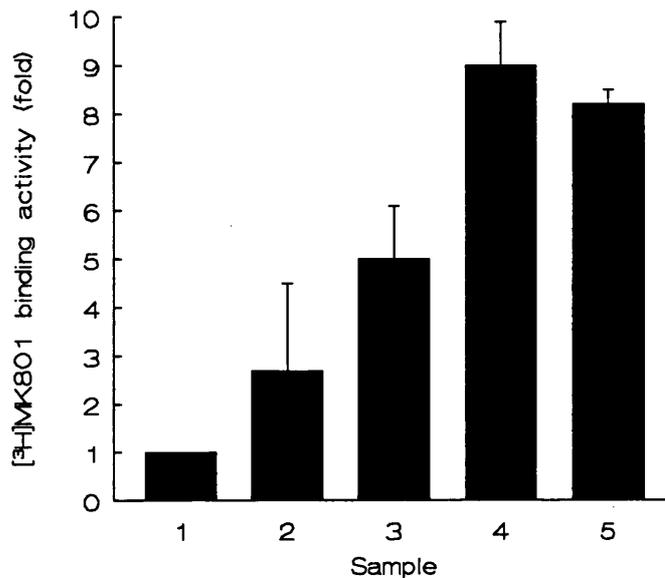


Figure 2.3.21 **The effect of varying the pCISNMDAR1-1a / pCISNMDAR2A ratio used for co-transfection on the expression of heteromeric receptors in HEK 293 cells**

HEK 293 cells were transfected with pCISNMDAR1-1a and pCISNMDAR2A in different ratios such that the total DNA used for transfection was always 10 μ g. Cells were cultured post-transfection in the presence of AP5 (200 μ M), harvested at 48 h and assayed for [³H]MK801 specific binding activity. Values are the means \pm S.D. for three separate experiments.

Lane 1 Cells were transfected with pCISNMDAR1-1a alone

Lanes 2-5 Cells were transfected with pCISNMDAR1-1a and pCISNMDAR2A in ratios: 1:1, 1:2, 1:3 and 1:5.

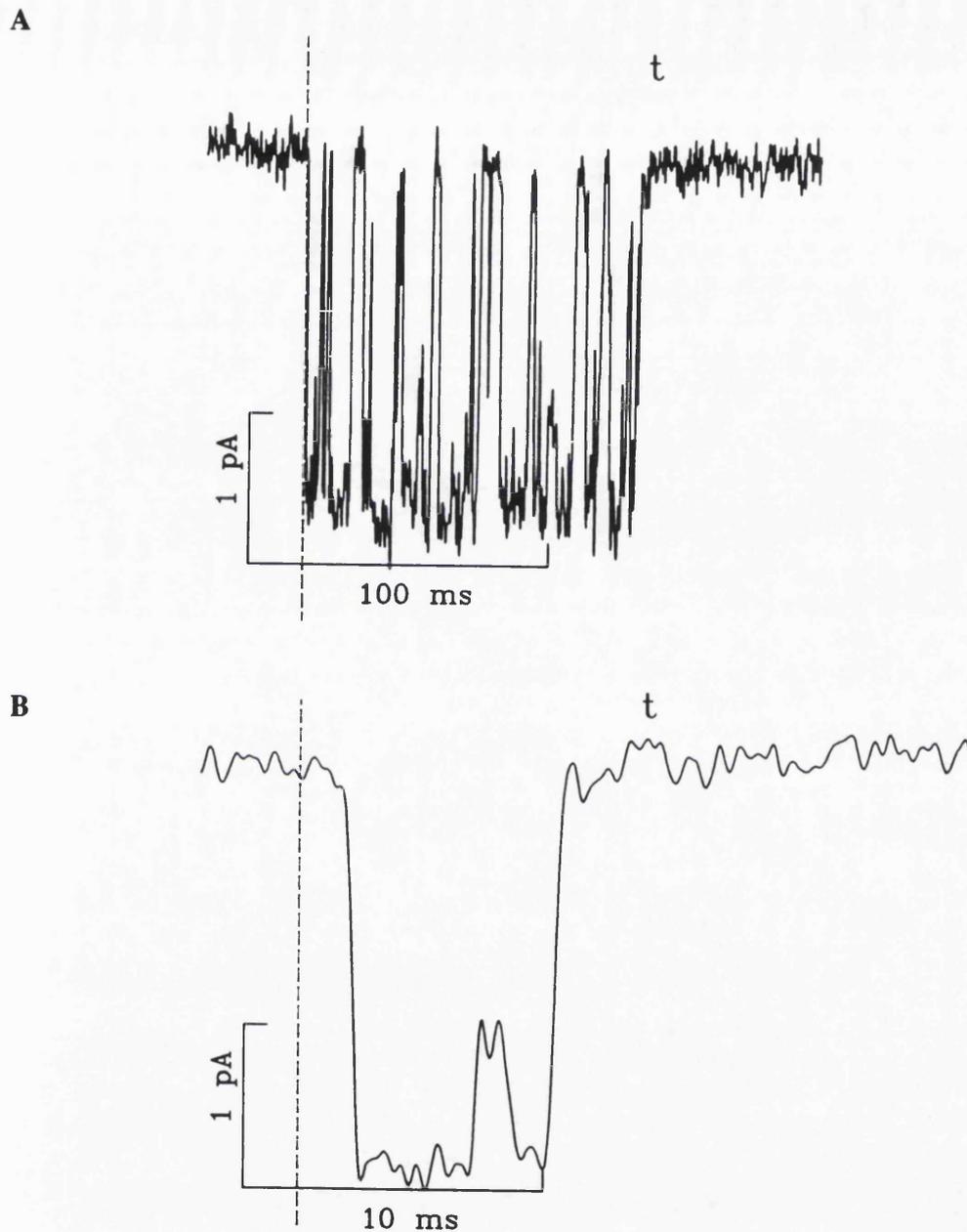


Figure 2.3.22 Single channel recording from an outside-out patch obtained from transfected HEK 293 cell.

A typical single channel recording of the NMDAR1-1a/NMDAR2A receptor-channel following application of 100 nM glutamate / 10 μ M glycine / 1 mM Ca^{2+} in the absence of Mg^{2+} . (A) Standard trace, (B) Expanded trace.

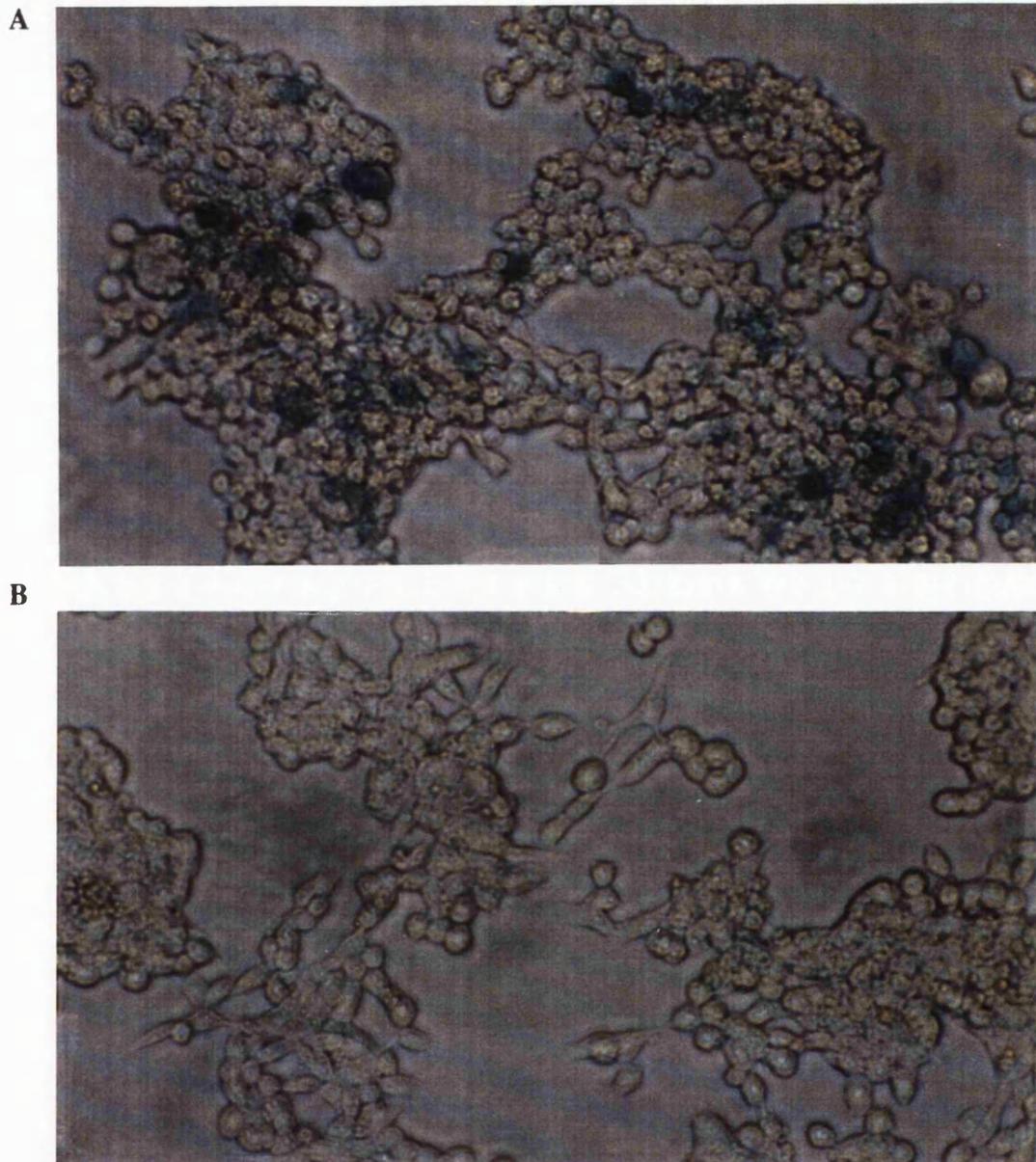


Figure 2.3.23 An example of cell survival following co-transfection with pSV- β -galactosidase, pCISNMDAR1-1a and pCISNMDAR2A

HEK 293 cells were co-transfected with pSV- β -galactosidase, the pCISNMDAR1-1a, the pCISNMDAR2A and cultured for 44 h in the presence (A) or absence (B) of AP5 and DKA (200 μ M each). Cells were stained with x-gal as described in 2.2.19. The photographs were taken on an inverted microscope and the film was developed by standard procedure.

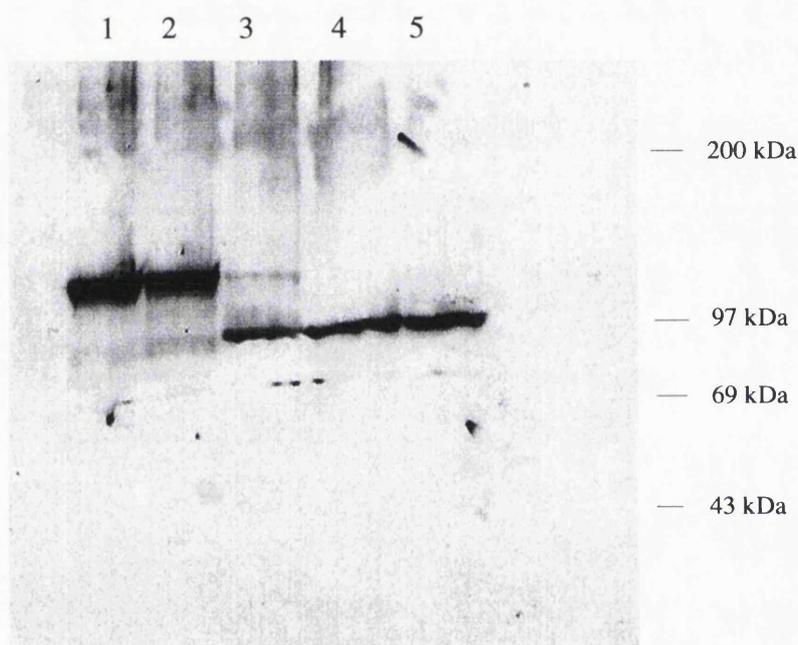


Figure 2.3.24 Dose-dependent effect of tunicamycin on glycosylation of the NMDAR1-1a polypeptide

HEK 293 cells were transfected with pCISNMDAR1-1a and subsequently grown in the presence of different concentrations of tunicamycin. In lanes 1-5 tunicamycin concentrations were 0.0 µg/ml, 0.02 µg/ml, 0.1 µg/ml, 0.5 µg/ml and 2 µg/ml. Cells were harvested 16 h post-transfection and analysed by immunoblotting.

Affinity purified antibody concentration was (10 µg/ml)

2.4 DISCUSSION

The properties of wild-type and mutant NMDA receptors have been characterized electrophysiologically following transient expression in the *Xenopus* oocytes or in mammalian cells (e.g. Mori *et al.*, 1992; Monyer *et al.*, 1992). For the characterization of the cloned NMDA receptors biochemically, higher receptor concentrations are required. To obtain such quantities of NMDA receptors, expression in mammalian cells was utilised. Shortly after the first NMDA receptor subunit was cloned (Moriyoshi *et al.*, 1991), we were kindly supplied with a cDNA encoding the rat NMDAR1-1a subunit. The first aim was to establish a model system for the study of the pharmacological and immunological properties of the recombinant receptor. It was already known that membrane-bound neurotransmitter receptors could be successfully expressed in HEK 293 cells using the CMV promoter/enhancer based vector (e.g. Werner *et al.*, 1991). Therefore, we chose the pCIS mammalian expression vector (2.1.4) and the HEK 293 cell line (2.1.2) as an experimental system. The subcloning strategy of the NMDAR1-1a cDNA into the pCIS was based on the available cloning sites in the pCIS vector and restriction sites 5' and 3' from the coding region in the cDNA. Also, attempts were made to reduce the 5' and 3' non-coding regions to minimum. Thus, the NMDAR1-1a cDNA was subcloned into the pCIS and a restriction enzyme analysis was performed to confirm the correct orientation of the cDNA (2.3.1). Following the cloning of the NMDAR2 subunits (NMDAR2A-R2D, e.g. Meguro *et al.*, 1992), M. Mishina kindly supplied us with the cDNA encoding the mouse NMDAR2A subunit. The first approach to subclone the NMDAR2A cDNA into the pCIS was unsuccessful (2.3.2). The reason for this remains unclear but most likely, it was due to the size of the NMDAR2A cDNA, 5.3 kb, which is larger than the size of the pCIS vector itself, 4.7 kb. Other laboratories have also had difficulties with subcloning of the NMDAR2 subunits into mammalian expression vectors (Monyer *et al.*, 1992).

The second strategy employed was based on a two step subcloning procedure (2.3.2). In the first step, cDNA encoding the initial 895 amino acids of the NMDAR2A subunit was subcloned into the *Xba I* / *Hind III* sites of the pCIS vector generating the pCISNMDAR2Ashort. In the same step, a *Hind III* site was engineered at the 3' end of of the NMDAR2A cDNA. This newly engineered *Hind III* site enabled the subcloning of the second part of the NMDAR2A subunit (*Hind III* / *Hind III*) into the pCISNMDAR2Ashort, thus generating the complete pCISNMDAR2A.

The pSV- β -galactosidase vector was used for the optimisation of transient expression in HEK 293 cells. Firstly, the important parameters for efficient transient expression such as the pH of the media and the cell number at the moment of transfection, were optimized (2.3.3). Following this optimisation, the NMDAR1-1a subunit was transiently expressed in HEK 293 cells. This was demonstrated by both [³H]MK801 specific binding activity and immunologically with anti-NMDAR1-1a antibodies. Homomeric NMDAR1 receptors were able to bind radioligand [³H]MK801 albeit with a low number of binding sites, suggesting a low efficiency of homomeric receptor assembly. It was found that in transfected HEK 293 cells, as in the rat brain, the NMDAR1-1a subunit is a N-glycosylated polypeptide with molecular weight of 117 000 daltons (2.3.3, Chazot *et al.*, 1992). A lower molecular weight immunoreactive species of M_r 97 000 was also detected on immunoblots. N-Deglycosylation of NMDAR1-1a polypeptides resulted in a single immunoreactive species of M_r 97 000, which was in good agreement with the predicted molecular weight of the NMDAR1-1a subunit and demonstrated that the two immunoreactive species detected on the immunoblot were the unglycosylated and glycosylated forms of the NMDAR1-1a subunit. In the NMDAR1 polypeptide, 10 potential N-glycosylation sites were predicted (Moriyoshi *et al.*, 1991). Thus, it was possible that due to overexpression of the NMDAR1-1a polypeptide in HEK 293 cells, N-glycosylation machinery was overloaded and unglycosylated peptides were also

detected by immunoblotting. The size of the NMDAR1-1a subunit was in good agreement with the identification of a M_r 120 000 polypeptide in the rat brain specifically photoaffinity-labelled with an azido derivative of MK801 (Sonders *et al.*, 1990). Similar results were reported recently, with monoclonal and polyclonal anti-NMDAR1-1a antibodies recognising immunoreactive species of M_r 117 000 in the rat brain and transfected cells (Brose *et al.*, 1993; Sucher *et al.*, 1993; Tingley *et al.*, 1993).

The recombinant pCISNMDAR2A was also transiently expressed in HEK 293 cells (2.3.4). Although the expression of the NMDAR2A subunit was demonstrated immunologically, no [3 H]MK801 specific binding activity was detected in transfected HEK 293 cell homogenates. This suggested that the NMDAR2A subunit alone was not able to form the functional homomeric channel, or that the [3 H]MK801 binding site is within the NMDAR1 subunit. It has been reported by other group that the expression of NMDAR2 subunits alone did not result in functional homomeric channels (Ishii *et al.*, 1993). On immunoblotting, a single immunoreactive species of M_r 180 000 was detected with anti-NMDAR2A antibodies. N-Deglycosylation of NMDAR2A polypeptides resulted in a single immunoreactive species of M_r 165 000 which was in good agreement with the predicted molecular weight from the cDNA. A large number of putative N-glycosylation sites was predicted at the extracellular regions of the NMDAR2A polypeptide (12 sites, Ishii *et al.*, 1993).

To compare the transfection efficiency between different cell lines, CHO cells were transiently transfected with the control vector pSV- β -galactosidase and the recombinant pCISNMDAR1-1a. Transfection with the control vector resulted in a lower transfection efficiency (5-10 %, 2.3.3). In comparison with immunoreactivity obtained with transfected HEK 293 cells, significantly lower immunoreactivity was detected in proteins from transfected CHO cells with anti-NMDAR1-1a antibodies. The lower immunoreactivity on the immunoblot could be explained by the lower transfection efficiency of CHO cells and with usage

of CMV promoter/enhancer based vector, pCIS, which is designed specifically for HEK 293 cells and may have a lower transcription efficiency in CHO cells.

In the co-transfection experiments cDNAs from two different species were used, a rat cDNA for the NMDAR1-1a subunit and a mouse cDNA for the NMDAR2A subunit. Since the rat and mouse NMDAR1-1a protein sequences are 99.8 % identical, with only two conserved substitutions in the C-terminal region, and the fact that the rat and mouse NMDAR2A protein sequences are more diverse, the heteromeric receptor can be referred to as the mouse NMDA receptor. The co-transfection of the pCISNMDAR1-1a and pCISNMDAR2A subunits under the standard transfection conditions resulted in aberrant cells and cell death (2.3.5). When co-transfected cells were grown in the standard culture medium, 22-24 % of the total cells in the culture flask died up to 24 h post-transfection (Table 2.3.3). As the cell transfection efficiency was estimated to be 20 ± 2 %, it was thought that all co-transfected cells died. The NMDA receptor complex is permeable to Ca^{2+} and Ca^{2+} influx under certain conditions can lead to neurotoxicity (Choi, 1988). The voltage-dependent Mg^{2+} block of the NMDA receptor has been well characterized and at the normal resting membrane potential (-80 mV), the NMDA receptor should be blocked with physiological Mg^{2+} concentration (e.g Mayer *et al.*, 1984). In the cell culture medium used for cotransfection experiments, the Mg^{2+} concentration was 0.7 mM, but the resting membrane potentials of HEK 293 cells were in the range of -15 to -50 mV (T. Smart, personal communication), potentials at which the voltage-dependent Mg^{2+} block is greatly reduced (Burnashev *et al.*, 1992b). Thus we proposed that the mechanism for observed cell death following co-transfection experiments, was the glutamate activation of heteromeric NMDA receptors and resultant Ca^{2+} influx into cells (Cik *et al.*, 1993).

Conditions were established for the optimum expression of heteromeric NMDA receptors in viable cells (2.3.4.1, Cik *et al.*, 1993). The presence of the NMDA receptor antagonist, AP5, in the culture medium post-transfection was

required for the prevention of the majority of cell death. Other laboratories have also employed AP5 for successful expression of heteromeric NMDA receptors in Xenopus oocytes (e.g. P. Stern, personal communication). Also, co-transfected cells were grown in the glutamine-free medium. Although we did not have direct evidence that the glutamine-free medium significantly increased cell viability, it has been reported that NMDA receptor-mediated damage of primary neuronal cells was dependent on the presence of glutamine in the culture medium (Driscoll *et al.*, 1993). Once the conditions were established for the expression of heteromeric NMDA receptors in viable cells, the optimum DNA ratio for the pCISNMDAR1-1a / pCISNMDAR2A was established (2.3.5.1.2). This was important to determine because of possible differences in efficiency of transcription and translation for different subunits and still unknown stoichiometry of the NMDA receptor. It was found that the optimum DNA ratio was 1:3 for the pCISNMDAR1-1a : pCISNMDAR2A. The time-dependence of the expression of the NMDAR1-1a and the NMDAR2A subunits was also investigated. The time course of the appearance of immunoreactive species and [³H]MK801 specific binding was followed for up to 62 h post-transfection. A similar time-dependent pattern for both immunoreactivity of singly transfected and co-transfected subunits and for [³H]MK801 specific binding activity was observed, with maximal effect observed at a time of 24 h post-transfection. Polypeptide expression was observed even after 3 h post-transfection (not shown). Thus routinely, co-transfected cells were grown in the presence of AP5 and assayed at the time of 24 h post-transfection. At that time point, [³H]MK801 specific binding activity was detected with up to a 10-fold increase in binding in comparison to HEK 293 cells expressing the NMDAR1-1a alone (Figure 2.3.15). When heteromeric NMDAR1-1a and NMDAR2A or NMDAR2C receptors were expressed in Xenopus oocytes, electrophysiological responses (peak amplitudes) were more than 10-fold larger than that obtained with homomeric assembly of the NMDAR1-1a, indicating that heteromeric receptor

formation was likely to form from the NMDAR1-1a and the members of NMDAR2 subunit family (e.g Ishii *et al.*, 1993). To confirm that HEK 293 cells express functional heteromeric NMDA receptors present at the surface of the cell, single-channel recordings in an outside-out patch were performed at the time point 20 h following cotransfection. The functional properties of the single-channels were essentially the same as the properties of the single-channels recorded in *Xenopus* oocytes expressing the NMDAR1-1a and the NMDAR2A subunits. Moreover these properties were very similar to native receptors in hippocampal neurons (Stern *et al.*, 1994a; Stern *et al.*, 1994b).

Although transfected cells were routinely grown in the presence of AP5, some cell death was still apparent. To further investigate prevention of cell death, the co-transfected HEK 293 cells were grown in the presence of one, or the combinations of two compounds, known to antagonise NMDA receptor activation. The effect of these compounds on cell death was monitored by trypan blue exclusion and the CytoTox 96TM assay (2.3.5.2). The advantages of using the CytoTox 96TM compared to the trypan blue exclusion method were in the number of samples that could be simultaneously processed within an experiment in a shorter time, and with a greater consistency of results between different experiments. The presence of AP5 and the antagonist of the strychnine-insensitive glycine site, DKA, resulted in no significant difference between the percentage of cell death in non-transfected and co-transfected HEK 293 cells. It is interesting to note that even the presence of DKA, could decrease cell death, which is in line with evidence that glycine is a co-agonist of the NMDA receptor (Kleckner and Dingledine, 1988). Recently, it has been reported that co-transfection of the NMDAR1-1a and the NMDAR2A resulted in cell death, except that in this preliminary report, cell death was not prevented by inclusion of the AP5 or DKA in the medium post-transfection except in mM concentration range (Anegawa *et al.*, 1993 and N.J. Anegawa personal communication). It was possible that in these experiments higher cell density was used and that due to

electrical coupling between cells, all cells died.

It was assumed that following co-transfection, all cells that were co-transfected died. To test this assumption, HEK 293 cells were co-transfected with the pSV- β -galactosidase and with the wild-type NMDAR1-1a/NMDAR2A subunits and subsequently grown in the presence or absence of AP5 and DKA (2.3.5.3). The percentage LDH release was assayed 20 h post-transfection and the percentage x-gal-stained cells were determined 44 h post-transfection in the same experimental flasks. No cells were stained for β -galactosidase activity when co-transfected cells were grown in the absence of the NMDA receptor antagonists and significant cell death was observed. When co-transfected cells were grown in the presence of AP5 and DKA, co-transfected cells were stained (~ 19 %), and no significant cell death was observed.

To investigate the effect of N-glycosylation on the assembly of heteromeric NMDA receptors, co-transfected HEK 293 cells were grown in a range of concentrations of tunicamycin, a selective inhibitor of asparagine-linked oligosaccharide synthesis (2.3.5). It was shown that the presence of tunicamycin in the culture medium post-transfection decreased [³H]MK801 specific binding activity. This effect was dose-dependent and it was due to a change in affinity of the receptor for the ligand rather than a decrease in total number of binding sites. Also, an increase in unglycosylated NMDAR1-1a subunit polypeptide on an immunoblot was observed. Although following the removal of tunicamycin from the media, [³H]MK801 specific binding activity returned to the control level, re-glycosylation of the NMDAR1-1a polypeptide occurred only partially. It has been reported that the precise nature of the oligosaccharide modification is not critical for the receptor function (Buller and White, 1990). As removal of tunicamycin resulted in partial reglycosylation, this was presumably sufficient to recover the binding of radioligand [³H]MK801 with high affinity. Site-directed mutagenesis of putative N-glycosylation sites could give definitive answer to the importance of each of 10 putative sites of the NMDAR1-1a subunit in receptor

assembly.

In summary, the NMDAR1-1a and the NMDAR2A cDNAs were successfully subcloned into the mammalian expression vector, pCIS. Optimal conditions for the transient expression of homo- and heteromeric NMDA receptors in HEK 293 cells were established. As co-expression of the NMDAR1-1a and the NMDAR2A resulted in receptor-mediated cell death, a study was made to establish conditions for the prevention against cell death by using different antagonists of the NMDA receptor. Only the presence of saturation concentrations of AP5 and DKA in the cell culture medium post-transfection resulted in the complete prevention of cell death.

CHAPTER 3

Site-directed, *in vitro* mutagenesis of the
NMDAR1-1a subunit

3.1 INTRODUCTION

3.1.1 SITE-DIRECTED *IN VITRO* MUTAGENESIS

Site-directed mutagenesis is a technique, which allows the creation of any predetermined change in DNA sequence. It can be used to examine gene regulation by altering the sequences which control transcription of DNA or by changing the protein coding region of specific genes, it can be used to study the structure-function relationships of proteins. The specific mutations in DNA sequence can be generated by using different approaches. The simplest way is to use restriction enzymes when the suitable sites are present in the DNA sequence to produce deletion mutants. The two-step PCR can also be used to produce specific point mutations (Ho *et al.*, 1989; e.g Mori *et al.*, 1992). But, the most widely used method is the oligonucleotide-mediated mutagenesis which can produce a broad range of specific mutations including: insertion mutations, deletion mutations and substitutions (point mutations) of single or multiple bases, for a known DNA sequence. Work in the 1970s on the single-stranded genome of the small bacteriophage, ϕ X174 led to recognition of the feasibility of the introduction of specific changes in DNA (e.g. Hutchison and Edgell, 1971). When the synthesis of oligonucleotides became routine and when high purity DNA-modifying enzymes were readily available, the first methods describing oligonucleotide-mediated mutagenesis were published (Hutchison *et al.*, 1978, Gillam and Smith, 1979). The methods in current use have in principle the same approach originally described by Hutchison and his colleagues but they are significantly more efficient in the generation of desired mutants. For the contribution in developing a method for the oligonucleotide-mediated mutagenesis, M. Smith received the Nobel prize in chemistry, in 1993.

One of the improved methods for the oligonucleotide-mediated mutagenesis was described by Zoller and Smith, (1984). Their method uses two

primers. The first primer is a mutant oligonucleotide, the second primer binds to the template DNA at the 5' side from mutant primer and it is phosphorylated. Extension of the second primer and ligation with the mutant primer reduces the possibility for removal of the mutant oligonucleotide by DNA polymerase. This method has increased the mutation efficiency, but still there is a requirement for screening of colonies by hybridization, to detect mutants. Another method currently in use employs specialized *E. coli* host (CJ236, *dut*⁻, *ung*⁻, F') which produces the single-stranded DNA with a small number of thymine residues replaced by uracil (Kunkel T.A., 1985; Bio-Rad Muta-Gene kit). When such ssDNA is repolymerised and transfected in wild-type *E. coli*, the uracil containing strand is degraded and progeny bacteriophages are derived from the mutated strand giving rise to mostly mutant progeny (up to 80 %). Because of the high efficiency, mutants can be analysed directly by DNA sequencing. The method described by Eckstein and his co-workers (Taylor *et al.*, 1985a; Nakamaye and Eckstein, 1986), gives an even higher mutation efficiency of up to 95 %. This method is used in the Amersham oligonucleotide-mediated mutagenesis system which is schematically represented in Figure 3.1.1. The system does not require a specialized vector and any plasmid-based vector with a single-stranded phage origin of replication can be used. The method employs strand-specific selection, which removes the unwanted non-mutant strand. It is based on an observation that certain restriction enzymes (*Nci I*, *Psi I*) cannot cleave phosphorothioate DNA (Taylor *et al.*, 1985b). When the mutant strand is extended in the presence of deoxy-cytidine-5'-[α S]thiotriphosphate (dCTP α S), resultant heteroduplex contains one phosphorothioate, mutant strand and one non-phosphorothioate strand, non-mutant strand. As a result, the restriction enzyme *Nci I*, nicks only the non-mutant, the non-phosphorothioate strand. Such nicks can then be used by exonuclease III to digest a large part of the non-mutant strand. The undigested part of the non-mutant strand serves as a primer for DNA polymerase. When the non-mutant strand is then repolymerised, the mutant

homoduplex is generated. The removal of remaining ssDNA after extension of the mutant oligonucleotide by filtration also reduces the non-mutant background. Because of the high efficiency of the method, colonies can be directly DNA sequenced to find the desired mutant. This method has been used successfully in many laboratories and it has proved to be useful for studying the structure-function relationships of ligand-gated ion channels. The channel domain, M2, of the neuronal nicotinic receptor, was mutated using the Amersham system and the effect of these mutations on desensitization of the receptor and on calcium permeability were analyzed (Revah *et al.*, 1991; Bertrand *et al.*, 1993). The Eckstein method was also used by the group of Mishina in Japan to study the effects of mutations on calcium permeability of the AMPA-selective glutamate channels (Mishina *et al.*, 1991). The same approach to produce point mutations was employed to analyze the structure-function relationships of the GABA_A receptor subunits (Wieland *et al.*, 1992).

3.1.2 DNA SEQUENCING

DNA sequencing is one of the most important techniques in molecular biology. With the development of the DNA sequencing methods it has become possible to analyse in detail, the structure and function of DNA molecules and to deduce protein sequences from the corresponding DNA sequence of genes or cDNAs. The two methods in current use for DNA sequencing were developed in the late 1970s (Sanger *et al.*, 1977; Maxam and Gilbert, 1977). Although these two techniques are different in principle, they both generate separate populations of radiolabelled oligonucleotides which are then resolved by vertical polyacrylamide gel electrophoresis under conditions that can discriminate between individual DNAs which differ in length by as little as one nucleotide. The Maxam-Gilbert method involves chemical degradation of the original, radiolabelled DNA molecule. This DNA is then partially cleaved in five separate

chemical reactions which are specific for one or two bases. The five separated radiolabelled populations are resolved by gel electrophoresis and the 3' labeled DNA molecules are then detected by autoradiography. Although the Maxam-Gilbert method is less used than the Sanger method, it has advantage that the DNA sequence is obtained from the original molecule and it can be used for the analysis of DNA modifications such as methylation and the interactions of proteins with DNA by chemical protection.

Sanger developed a method in which dideoxynucleoside 5'-triphosphates (ddNTPs) are used to terminate extension of primed, ssDNA (Sanger *et al.*, 1977). Four separate enzymatic reactions are used, each with one of the four ddNTPs, which will specifically terminate the DNA polymerase reaction at positions occupied by every G, A, T or C in the DNA template. The four different radiolabelled oligonucleotides pools are analysed by gel electrophoresis and the DNA sequence read from the autoradiography image. Several modifications of the original Sanger method are now currently in use. They differ in the DNA polymerase used for the extension of primed ssDNA. The original Sanger's method used the Klenow fragment of DNA Polymerase I. This enzyme is still in use but it has low processivity, a low rate of polymerization and it does not copy efficiently regions with secondary structures. The second enzyme in use is Taq DNA polymerase. This enzyme works efficiently at 70-75°C, thus eliminating the problem of extension of secondary structures. Taq DNA polymerase also has a high degree of processivity. Sequenase (Tabor and Richardson, 1987), and Sequenase version 2.0 (Tabor and Richardson, 1989) are forms of bacteriophage T7 DNA polymerase that were modified to eliminate 3'→5' exonuclease activity. Sequenase is a chemically-modified enzyme and Sequenase version 2.0 is recombinant enzyme that is more stable and has a higher specific activity than the chemically modified Sequenase. The sequencing reaction using Sequenase is set up in two-steps to take advantage of the high processivity rate of enzyme. In the first step, low concentration of dNTPs are

used to ensure efficient incorporation of radiolabelled [$\alpha^{35}\text{S}$]dATP. In the second step, the reaction is divided into a set of four reactions, each with one of the four ddNTPs. In this step, polymerization continues until a chain-terminating nucleotide (ddNTP) is incorporated into the growing chain. Two different radionucleotides are used in the sequencing reaction, [$\alpha^{32}\text{P}$]dATP and [$\alpha^{35}\text{S}$]dATP. [$\alpha^{35}\text{S}$]dATP has some advantages: it is safer, it gives sharper bands in the autoradiography and it has a longer half-life.

In this chapter, the generation of mutant NMDAR1-1a subunits will be described. It has been shown that the point mutation (N598Q) reduced the Ca^{2+} -permeability of cloned NMDA receptors (Burnashev *et al.*, 1992b). The effect of the (N598Q) mutation and mutations of acidic amino acids in the region adjacent to TM2 of NMDAR1-1a, on receptor-mediated cell death, will be described. The rationale for the latter mutations was that it has been described that a carboxyl group is important for the binding of [^3H]MK801 to native, membrane-bound and recombinant, heteromeric NMDA receptors (Chazot *et al.*, 1993; Chazot *et al.*, 1994a). Thus chemical modification of carboxyl groups, by treatment of membranes with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) resulted in the inhibition of specific [^3H]MK801 binding activity. This inhibition was protected by preincubation of brain membranes and HEK 293 cell homogenates with Mg^{2+} ($\text{EC}_{50} = 1.3 \text{ mM}$).

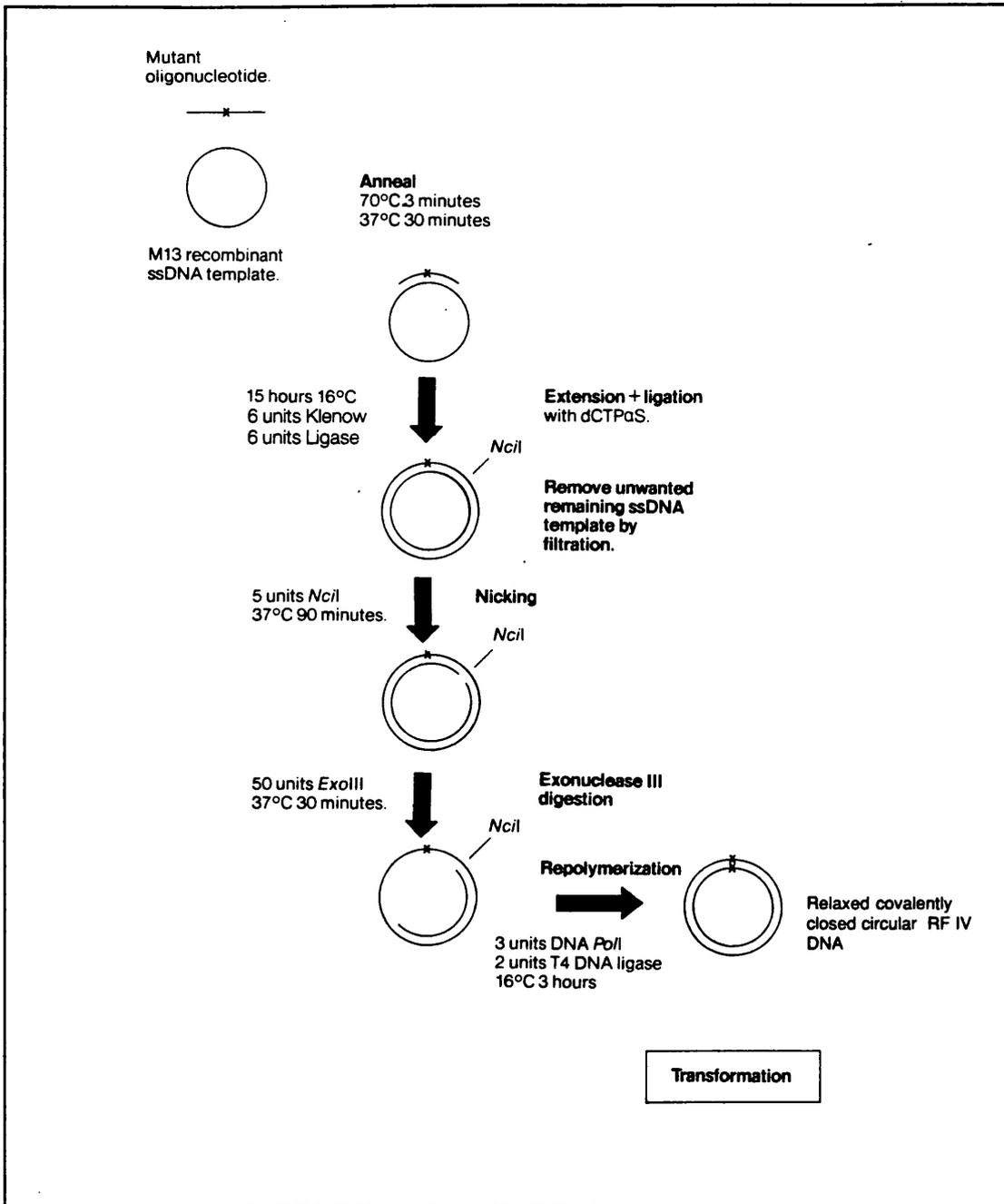


Figure 3.1.1 The Amersham oligonucleotide-directed *in vitro* mutagenesis system.

(From Oligonucleotide-directed *in vitro* mutagenesis system, version 2.0, Amersham International plc)

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Oligonucleotide-directed *in vitro* mutagenesis system (version 2.1) and [³⁵S] deoxy-adenosine-5'-[αS]thiotriphosphate (dATPαS, >1000 Ci/mmol) were obtained from Amersham International plc (Buckinghamshire, UK). Helper phage, M13KO7 was purchased from Pharmacia Biotech Ltd. (Herts, UK). T4-polynucleotide kinase, isopropyl-β-D-thiogalactopyranoside (IPTG), and deoxy-adenosine-5'-triphosphate (dATP) were obtained from Boehringer Mannheim UK (East Sussex, UK). Oligonucleotide probes were purchased from Oswell DNA Service (Edinburgh, UK). Bio-spin 30 chromatography columns were obtained from Bio-Rad Laboratories Ltd. (Hertfordshire, UK). Sequenase[®], DNA sequencing kit (version 2.0) was purchased from Cambridge BioScience (Cambridge, UK). X-OMAT AR-5 Kodak scientific imaging film and urea were obtained from Sigma Chemical Co Ltd. (Poole, Dorset, UK). Acrylamide, N'N'-methylenebisacrylamide and Eppendorf GELoader capillary tips were purchased from BDH Laboratory Supplies (Leicestershire, UK). Thin-walled GeneAmp[™] reaction tubes were purchased from Perkin-Elmer Ltd. (Buckinghamshire, UK). All other materials were described in Chapter Two or were from commercial sources.

Strains: E. Coli strain NM522 (Genotype: supE, thi, Δ(hsdMS-mcrB)5, Δ(lac-proAB), F'[proAB⁺, lac^q, lacZΔM15]) was obtained from Pharmacia Biotech Ltd. (Herts, UK).

E. Coli strain TG1 (Genotype: K12, Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA⁺B⁺, lac^q, lacZΔM15) was supplied with oligonucleotide-directed *in vitro* mutagenesis system (version 2.1), obtained from Amersham International plc (Buckinghamshire, UK).

3.2.2 PREPARATION OF SS PLASMID DNA

The ssDNA was prepared according to Sambrook *et al.*, (1989) and Vieira and Messing, (1987). A single colony of *E. coli* strain NM522 harbouring the plasmid, was transferred from the LB plate to 2 ml 2 x YT medium (tryptone 16 g/l, yeast extract broth 10 g/l, NaCl 5g/l), containing 100 µg/ml ampicillin and 0.001 % (w/v) thiamine, in a loosely capped 50 ml Falcon tube. The culture was incubated at 37°C with shaking. When the culture reached saturation (3-5 h), an aliquot (100 µl) was transferred to a Falcon tube with 10 ml 2 x YT medium containing 4×10^8 pfu/ml helper phage M13KO7 and incubated for 1 h at 37°C. The antibiotic, kanamycin, was added to a final concentration of 70 µg/ml and the culture was incubated overnight. Following this overnight incubation, the culture was aliquoted into microfuge tubes and centrifuged at 12 000 x g for 5 min at 4°C. The supernatant was transferred to a fresh tube and recentrifuged until the pellet was not visible (2-3 times). Each tube (1.2 - 1.3 ml of the supernatant) was mixed with 200 µl of 20 % (w/v) PEG in 2.5 M NaCl, incubated at room temperature for 15 min and centrifuged at 12 000 x g for 10 min at 4°C. The pellet was resuspended in 100 µl of TE buffer (2.2.5.3) and extracted with 50 µl of phenol, followed by extraction with 90 µl of chloroform (2.2.5.2). The upper phase (70 µl) was transferred to microfuge tube containing 300 µl of 25:1 mixture of absolute ethanol and 3M Na-acetate, pH 5.2, stored for 30 min at -70°C and centrifuged at 12 000 x g for 10 min at 4°C. The pellet was washed with 70 % (v/v) ethanol, dried for 10 min in open air and resuspended in 50 µl of TE buffer. The ssDNA concentration was determined as in 2.2.7 and adjusted to 1 µg/µl.

3.2.3 SMALL-SCALE PREPARATION OF PLASMID DNA FOR THE DNA SEQUENCING REACTION

The method for small-scale plasmid preparation was essentially as in 2.2.5.1-3, with the following changes, as adapted by Kraft *et al.*, (1988). A single colony of *E. coli* containing the correct plasmid, was transferred from the LB plate stock to 10 ml LB medium (2.2.2) containing 100 µg/ml ampicillin in a loosely capped 50 ml Falcon tube. The culture was incubated overnight (20-24 h) at 37°C with shaking (200 rpm). The overnight culture (1.5 ml) was centrifuged at 12 000 x g for 3 min at room temperature. The supernatant was removed by aspiration and an additional 1.5 ml of culture was added, the sample was re-centrifuged for 30 sec and re-aspirated. The pellet was resuspended in 100 µl ice-cold TGE buffer (2.2.5.1), by pipette suction. To each tube, 200 µl freshly prepared alkaline SDS solution (2.2.5.1) were added and the contents were mixed by inverting the tube rapidly five times. Tubes were stored on ice. To each, 150 µl ice-cold 3 M potassium acetate, pH 4.8 were added and the tubes were mixed by inversion. The tubes were kept on ice for 5 min, centrifuged at 12 000 x g for 10 min at 4°C and the supernatants were transferred to fresh tubes. RNase A was added to a final concentration of 50 µg/ml and tubes were incubated at 37°C for 30 min. The supernatants were extracted with an equal volume of the phenol:chloroform mixture (phenol: chloroform:amyl alcohol, 25:24:1, volume ratio) and centrifuged at 12 000 x g for 1 min at room temperature.

The dsDNA was precipitated with 2.5 volumes 100 % ethanol for 30 min at -70°C and pelleted at 12 000 x g for 10 min at 4°C. The supernatants were removed and the pellets were washed with 1 ml 70 % (v/v) ethanol. The plasmid DNA pellets were dried in a vacuum desiccator for 15 min and dissolved in 16.8 µl TE buffer. On ice, 3.2 µl 5 M NaCl were added and mixed, followed by addition of 20 µl 13 % (w/v) PEG, mixed and incubated on ice in a cold room for 30 min. The samples were centrifuged for 10 min at 4°C and the supernatants

were discarded. The pellets were washed with 70 % (v/v) ethanol, dried and dissolved in 20 μ l sterile water.

3.2.3.1 Denaturing of dsDNA template

To the DNA samples from the previous step, 2 μ l a freshly prepared solution of 2 M NaOH and 2 mM EDTA were added, mixed and incubated for 5 min at room temperature. Samples were placed on ice and 8 μ l 1 M Tris-HCl, pH 4.5 and 3 μ l 3 M Na-acetate were added sequentially and mixed. Ice-cold absolute ethanol (75 μ l) was added and mixed, and tubes were incubated on dry ice for 20 min, followed by centrifugation at 4°C for 5 min. The pellets were washed with 70 % (v/v) ethanol, dried in vacuum desiccator and used for dideoxy sequencing of ds plasmid DNA as described in 3.2.5.

3.2.4 OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS REACTION

The mutagenesis reaction was performed as recommended in protocols supplied by the manufacturer.

3.2.4.1 Design of the oligonucleotides for mutagenesis and sequencing

Oligonucleotides were designed by using the PCRPLAN, as part of the PC/GENE software package (IntelliGenetics, Inc., California, USA). The program parameters were defined as follows: the melting temperature (T_m) was calculated by method of Rychlik *et al.*, (1990), with the acceptable range of melting temperatures of $\pm 15^\circ\text{C}$; the range of optimal primer lengths was 15-25 nucleotides; the range of acceptable GC percentage was 40-60 %; the two bases at the 3' had to be G or C to bind primer more tightly to the template and

prevent non-specific base pairing; four self complementary bases were maximum for primer self complementarity (primer to primer binding) and for stem size in stem-loop formation (primer binds to itself); maximum percentage of bases complementary to other template region was 70 %.

The following oligonucleotides were designed by the PCRPLAN;

ss sequencing primer, used for sequencing of ss plasmid DNA, it would prime sequencing of + strand, 20 bases.

5' GAAACCAGCCCACACCATGC 3'

ds sequencing primer, used for sequencing of ds plasmid DNA, it would prime sequencing of - strand, 23 bases.

5' GACAGGACCATTGTTCAAGAGGG 3'

mutant (N598Q), oligonucleotide, 22 bases, changes underlined.

Mutation					Q				
Protein	G	V	L	L	N	S	G	I	G
+ Template	GGC	GTC	CTG	CTC	AAC	TCC	GGC	ATT	GGG
Mutant 1	3' G	CAG	GAC	GAG	<u>GTT</u>	AGG	CCG	TAA	5'

mutant (E603A), oligonucleotide, 19 bases, changes underlined, GC percentage was not within acceptable range (74 %), primer has a self complementarity, primer forms a stem-loop, a single base repeat containing 4 bases was present.

Mutation				A						
Protein	G	I	G	E	G	A	P	R	S	F
+ Template	GGC	ATT	GGG	GAA	GGT	GCC	CCC	CGG	AGT	TTC
Mutant 2a	3' CG	TAA	CCC	<u>CGT</u>	CCA	CGG	GG	5'		

mutant (E603V), oligonucleotide, 27 bases, changes underlined, primer length was longer than optimal, GC percentage was not within acceptable range (67 %), a single base repeat containing 6 bases was present.

Mutation				V							
Protein		G	I	G	E	G	A	P	R	S	F
+ Template		GGC	ATT	GGG	GAA	GGT	GCC	CCC	CGG	AGT	TTC
Mutant 2	3'	CG	TAA	CCC	<u>CAT</u>	CCA	CGG	GGG	GCC	TCA	A 5'

mutant (EED579-581QQN), oligonucleotide, 24 bases, changes underlined.

Mutation				Q	Q	N					
Protein			E	E	E	E	D	A	L	T	L
+ Template		G	GAG	GAG	GAG	GAA	GAT	GCA	CTG	ACC	CTG
Mutant 3	3'	C	CTC	CTC	<u>GTC</u>	<u>GTT</u>	<u>TTA</u>	CGT	GAC	TG	5'

mutant (EEEEED576-581QQQQN), oligonucleotide, 36 bases, primer length was longer than optimal.

Mutation				Q	Q	Q	Q	Q	N						
Protein			S	E	E	E	E	E	D	A	L	T	L		
+ Template		AAG	GTG	AAC	AGT	GAG	GAG	GAG	GAG	GAA	GAT	GCA	CTG	ACC	CTG
Mutant 4	3'	C	CAC	TTG	TCA	<u>GTT</u>	<u>GTC</u>	<u>GTC</u>	<u>GTC</u>	<u>GTT</u>	<u>TTA</u>	CGT	GAC	TG	5'

3.2.4.2 Purification of the mutant oligonucleotide

The mutant oligonucleotides longer than 20 bp were purified using Bio-Spin 30 columns as recommended by the manufacturer. The excess buffer from the column was drained by gravity and discarded. To exchange buffer, 300 µl of TE buffer was applied to the column and drained by gravity. The column was centrifuged for 2 min at 1100 x g to remove excess buffer. The sample (100 µl) was applied very carefully, directly to the centre of the column allowing the liquid to drain into the gel bed between successive drops of the sample. The column was connected to the collection tube and centrifuged for 4 min at 1100 x g at 4°C The oligonucleotide concentration was determined as described in 2.2.7.

3.2.4.3 Phosphorylation of the mutant oligonucleotide

In a microfuge tube, 5 OD_{λ=260} units/ml of the mutant oligonucleotide (2.5 μl), was mixed with 2.5 μl of water and 3 μl 10 x kinase buffer (1 M Tris-HCl, pH 8.0, containing 100 mM MgCl₂, 70 mM DTT and 10 mM dATP). To the mixture, 2 units of T4 polynucleotide kinase were added, mixed gently and incubated at 37°C for 15 min, followed by incubation at 70°C for 10 min. The phosphorylated oligonucleotides were used immediately.

3.2.4.4 Annealing of the mutant oligonucleotide to the ssDNA template

The ssDNA (5 μl, concentration 1 μg/μl), prepared as described in 3.2.2 was placed in a microfuge tube and mixed with the phosphorylated mutant oligonucleotide (2.5 μl, 1.6 pmol/μl), buffer 1 (3.5 μl) and 6 μl of water. The tube was incubated at 70°C for 3 min followed by incubation at 37°C for 30 min and placed on ice.

3.2.4.5 Synthesis and ligation of the mutant DNA strand

To the annealing reaction from the previous step, the next solutions were added; 5 μl of MgCl₂, 19 μl of nucleotide mix 1, 6 μl of water, 1.5 μl (6 units) of Klenow fragment and 3 μl (6 units) of T4 DNA ligase. The tube was incubated at 16°C overnight (16 h). To the reaction, 170 μl of water and 30 μl of 5 M NaCl were added and mixed by pipetting. To remove the ss (not filled and ligated, non-mutant) DNA, the sample was applied to the top half of the disposable centrifugal filter unit and centrifuged at 500 x g for 10 min at room temperature. The filter unit was washed with 100 μl 500 mM NaCl and centrifuged for 10 min. The DNA in the lower part of the filter unit was precipitated by adding 28 μl 3 M Na-acetate and 700 μl cold (-20°C) ethanol.

The tube was incubated in a dry ice/ethanol bath for 20 min and centrifuged at 4°C for 15 min at 12 000 x g. The pellet was washed with cold (-20°C) 70 % (v/v) ethanol, dried in the open air and resuspended in 25 µl buffer 2.

3.2.4.6 Nicking and digestion of non-mutant strand

The filtered sample (10 µl), from the previous step, was transferred to a fresh tube and mixed with 65 µl buffer 3 and 1 µl (5 units) of *Nci I* restriction enzyme. The sample was incubated at 37°C for 90 min. After incubation, during which nicking of the non-mutant strand has occurred, 66 µl of sample was mixed with 12 µl 500 mM NaCl, 10 µl buffer 4 and 2 µl (50 units) exonuclease III. Digestion of the non-mutated strand was performed at 37°C for 14 min and enzyme was inactivated by incubation at 70°C for 15 min.

3.2.4.7 Repolymerization and ligation of gapped DNA

To repolymerize and ligate gapped mutant DNA, the following was added to the reaction tube from the previous step: 13 µl nucleotide mix 2.5 µl MgCl₂ solution, 1 µl (3 units) DNA polymerase I and 1 µl (2 units) T4 DNA ligase. The sample was incubated at 16°C for 3 h, followed by DNA precipitation with one volume of 4 M ammonium acetate and four volumes of absolute ethanol. DNA was pelleted by centrifugation at 12 000 x g at room temperature and the pellet was washed with 70 % (v/v) ethanol. The pellet was dried and resuspended in 100 µl TE buffer.

3.2.4.8 Transformation of competent cells

Competent TG1 cells were transformed with reaction mixtures as described in 2.2.4. The control mutagenesis reaction, with "white" control M13 template and "blue" control oligonucleotide was transformed as described in 2.2.4 and plated out as follows. To the transformed cells, fresh plating mixture was added (200 μ l log phase *E. coli* cells, 40 μ l 100 mM IPTG and 40 μ l 2 % (w/v) x-gal). To the tube, 3-4 ml of molten H top agar (tryptone 10 g/l, NaCl 8 g/l and agar 8 g/l, kept at 45°C) was added, mixed by rolling and poured onto a prewarmed H plate (tryptone 10 g/l, NaCl 8 g/l, agar 15 g/l and ampicillin 100 μ g/ml). Plates were inverted and incubated at 37°C overnight. The ampicillin resistant colonies (3-4 for each sample) were analyzed by dideoxy sequencing.

3.2.5 DIDEOXY SEQUENCING REACTION

The dideoxy sequencing of ss and ds plasmid DNA was performed as recommended by the manufacturer. The annealing of the sequencing primer to ss or ds template was essentially the same. For each set of four sequencing lanes, a single annealing reaction was used. In a microfuge tube (0.5 μ l, thin-walled), primer (1 μ l, 1 pmol/ μ l) was mixed with reaction buffer (2 μ l) and DNA template (7 μ l, 1-2 μ g of the ss DNA template or the pellet of denatured ds DNA template from step 3.2.3.1 was resuspended in 7 μ l and used in the annealing reaction). A tube was incubated at 65°C for 2 min, then slowly cooled to <35°C over 30-40 min and stored on ice. While the annealing reactions were cooling, the termination mixtures (2.5 μ l of G, A, T and C mixtures), were dispensed in 0.5 μ l tubes and kept at 6°C. To the ice-cold annealing reactions, the following were added; 1 μ l 100 mM DTT, 2 μ l diluted labeling mix (diluted 5-fold with water), 0.5 μ l [³⁵S] dATP and 2 μ l diluted Sequenase polymerase (diluted 8-fold with enzyme dilution buffer). The labelling reaction was mixed and incubated for

3 min at room temperature. An aliquot (3.5 μ l) was transferred to each prewarmed (37°C for 1 min) termination tube, mixed and incubated at 37°C for 5 min. The reaction was stopped by the addition of 4 μ l stop solution and tubes were stored on ice. All incubations at temperatures different from room temperature, were performed using RoboCycler™ 40 (Stratagene, La Jolla, CA, USA).

3.2.5.1 Denaturing gel electrophoresis

All denaturing gel electrophoresis of sequencing samples were run on Bio-Rad Sequi-Gen™ cell, with 21 x 50 cm size of siliconized integral plate/chamber and machined wedge spacers (0.25-0.75 mm thickness). The gel (6 % (w/v) acrylamide/bis-acrylamide, 7 M urea) was prepared 20 h prior to use and pre-run for 2 h at 45°C (35W) in 1 x TBE buffer. The sequencing samples were denatured at 75°C for 2 min and loaded on the gel (1.8 μ l) using an Eppendorf digital ultra micro pipette and GELoader, capillary tips. The gel was run at 50°C (45W) until the bromophenol blue ran to the bottom of the gel. When two loadings were used, the gel was run firstly until the xylene cyanol FF ran to the bottom of the gel, then the gel was loaded a second time and run until bromphenol blue ran to the bottom of the gel. The Sequi-Gen™ cell was dismantled, the outer plate with gel was removed and placed in the soaking solution (5 % (v/v) acetic acid and 15 % (v/v) methanol) and soaked for 1 h to remove the urea. The gel was transferred to 3MM filter paper, dried at 80°C for 2 h and exposed to the Kodak X-OMAT AR-5 film for 36 h. Following exposure, the film was developed for 1 min using Kodak D-19 developer and fixed for 2 min using Kodak unifix.

3.3 RESULTS

3.3.1 OLIGONUCLEOTIDE-MEDIATED MUTAGENESIS OF THE NMDAR1-1a SUBUNIT

Most of the oligonucleotide-mediated mutagenesis methods require ssDNA. Such a DNA can be obtained by subcloning the target DNA into the M13 vector or into a plasmid vector with ssDNA phage origin of replication. The subcloned segment of target DNA should be as small as possible and preferably < 1 kb. This is because as the size of the target DNA increases, the chance that the mutagenic oligonucleotide will hybridize non-specifically increases. Shorter target DNA sequences also reduce the time required to sequence the target DNA after the mutagenesis reaction to confirm the mutation. To satisfy this criteria, the part of the NMDAR1-1a sequence (between two *Bgl II* sites, 1 600 bp, Figure 3.3.3) was subcloned into the pCIS vector which is capable of producing a ssDNA. Following the mutagenesis reaction, mutant *Bgl II / Bgl II* fragment was subcloned back into the pCISNMDAR1-1a. The strategy for the mutagenesis reaction is schematically shown in Figure 3.3.1.

3.3.1.1 Subcloning of the *BglII/BglII* fragment of the NMDAR1-1a subunit into the pCIS vector

The *BglII / BglII* fragment (1.6 kb) was excised from the plasmid pCISNMDAR1-1a (Figure 2.3.4) and cloned in both orientations, into the *Bam HI* site of the pCIS mammalian expression vector (Figure 2.3.2).

The plasmid pCIS was linearised with *Bam HI* enzyme, the cohesive termini were dephosphorylated as described in 2.2.8 and the linearised, dephosphorylated DNA molecule was purified using the Magic Clean-up resin (2.2.10). The plasmid pCISNMDAR1-1a was cut with *Bgl II* enzyme and the

resulting fragments were separated on the agarose gel (2.2.9). The 1.6 kb fragment was excised from the agarose gel and purified on the QIAEX matrix (2.2.13). The ligation reaction was set up as described in 2.2.9 and the competent *E. coli* (NM522 strain) were transformed as described in 2.2.4. The recombinants were analysed with *Eco RI* enzyme to confirm the orientations of the *BglIII/BglIII* fragment. The result is shown in Figure 3.3.2 with the recombinant pCISBglIII in sense orientation represented in lines 8 and 9.

3.3.1.2 Mutagenesis reaction

The mutagenesis reactions were carried out with the mutagenic oligonucleotides (N598Q, E603V, EED579-581QQN and EEEEEED576-581QQQQQN) as described in detail in 3.2.4.1-8. The positions of the mutations are shown in Figure 3.3.3. The control mutations with the "white" control template and the "blue" control oligonucleotide, supplied by the manufacturer, showed 79 % and 95 % mutation efficiency in two experiments respectively. Each stage of the mutagenesis reaction was analysed by taking a small sample at each step of the reaction, as schematically represented in Figure 3.3.4 and the result of the analysis is shown in Figure 3.3.5. As all controls of the mutagenesis reaction were indicating a successful experiment, the single colonies were grown overnight and small-scale ss and ds plasmid DNAs (3.2.2-3) were prepared for DNA sequence analysis.

3.3.2 DIDEOXY SEQUENCING OF PUTATIVE MUTANTS

For the Sanger dideoxy chain termination sequencing reaction, two sequencing primers were used (ds and ss primer, 3.2.4.1). Sequencing reactions were performed as described in 3.2.5. The four colonies from the putative (N598Q) mutant plate were analysed by using ss primer and ssDNA template.

The two samples had introduced the specific mutation in their coding regions (N598Q mutation) with no other changes in the sequenced coding regions (182 nucleotides). To confirm the specificity of the mutation, the sequencing reaction was repeated with ss and ds primers (using ss and ds DNA template). The double loading of the sequencing gel was used to extend readable DNA sequence. The checked region (281 nucleotides upstream from the ss primer and 341 nucleotides downstream from the ds primer) did not show any nonspecific change in the coding region. The three colonies from each putative E603V, EED579-581QQN and EEEEEED576-581QQQQN1E, mutants were grown overnight and ds DNA templates (3.2.3) were sequenced by using ss primer. For each of the putative mutants, two out of three samples had introduced the specific mutation, with no other changes in the sequenced coding region. The checked region was 294, 300 and 170 nucleotides upstream from the ss primer for E603V, EED579-581QQN and EEEEEED576-581QQQQN mutants respectively. The eight colonies of the putative mutant E603A were sequenced and no specific mutation was observed in any of them. The mutated *BglIII/BglIII* fragments were subcloned back to the pCISNMDAR1-1a recombinant in the correct orientation.

3.3.3 SUBCLONING OF THE MUTATED *BglIII/BglIII* FRAGMENTS INTO THE pCISNMDAR1-1a

The subcloning of the *BglIII/BglIII* fragment into the *Bam HI* site of the pCIS plasmid destroyed both of these two restriction enzymes sites, but the resulting GGATCT and AGATCC sites could be cut with the *Xho II* restriction enzyme leaving compatible ends to the *Bgl II*. Thus, mutated *BglIII/BglIII* fragments were cloned back into the *Bgl II* site of pCISNMDAR1-1a, without any changes in DNA sequence.

As the *Xho II* enzyme cuts pCISBglII at 9 sites, it would be difficult to separate the mutant *BglII/BglII* fragment from the resultant mixture. To overcome that problem, the mutant-pCISBglII was cut by *Cla I* and *Hind III* enzymes which have single recognition sites within the multiple cloning site of the pCIS vector (Figure 3.3.1). The mutant *BglII/BglII* fragments were rescued from the agarose gel and purified by using the QIAEX matrix (2.2.13). The purified mutant *BglII/BglII* fragments were then cut with *Xho II* and purified on the Magic Clean up column (2.2.10). The recombinant pCISNMDAR1-1a was linearised with *Bgl II*, the pCISNMDAR1-1a-linear was then separated from the *BglII/BglII* fragment on an agarose gel, cut out and purified by using the QIAEX matrix. The ligation reactions with the pCISNMDAR1-1a-linear and the mutant *BglII/BglII* fragments were set up as described in 2.2.9. To confirm the correct orientation of the mutant *BglII/BglII* fragments, restriction enzyme digestion with *Eco RI* were performed. Figure 3.3.6 shows the results obtained with pCISNMDAR1-1a (N598Q) mutant after *Eco RI* digestion and agarose gel analysis, with the pCISNMDAR1-1a (N598Q) containing mutant *BglII/BglII* fragment in the sense orientation represented in lanes 5, 7 and 8. The same subcloning results were obtained with the mutants pCISNMDAR1-1a (E603V), pCISNMDAR1-1a (EED579-581QQN) and pCISNMDAR1-1a (EEEEED576-581QQQQQN).

3.3.4 THE EFFECT OF MUTATIONS ON HEK 293 CELL SURVIVAL FOLLOWING CO-TRANSFECTION WITH THE MUTANT pCISNMDAR1-1a AND THE WILD-TYPE pCISNMDAR2A

In the previous chapter, the optimisation of conditions for the expression of both the NMDAR1-1a and the NMDAR2A subunits was shown (2.3.4). As co-transfection resulted in receptor-mediated cell death, the presence of the NMDA receptor antagonist, AP5, was essential for cell viability and a functional response to be observed. To further characterize the hypothesis of receptor-mediated cell death, a series of mutations in the NMDAR1-1a subunit was constructed, as described in 3.2.4 and 3.3.1. Thus, the HEK 293 cells were transfected by the calcium phosphate method (2.2.17), with the combination of one of the NMDAR1-1a mutants and with the wild-type NMDAR2A. The percentage of cell death post-transfection was compared with wild-type heteromeric NMDAR1-1a/NMDAR2A receptors. The percentage cell death was determined 20 h post-transfection using the CytoTox 96TM cytotoxicity assay (2.2.20), and results are summarised in Table 3.3.1. Differential effects on receptor-mediated cell death were observed following expression of mutant heteromeric receptors. The expression of the mutant polypeptides was verified by immunoblotting using anti-NMDAR1-1a antibodies (results not shown) and by [³H]MK801 radioligand binding activity (3.3.5). Following expression of the mutant NMDAR1-1a(N598Q) or NMDAR1-1a(E603V) subunits with the wild-type NMDAR2A subunit, a significant reduction in cell mortality was observed. Cell mortality was decreased to 50 % (P < 0.01) and to 45 % (P < 0.001) for the N598Q and E603V mutants respectively. The mutation EED579-581QQN decreased cell mortality to 81 % (P < 0.02) and the mutation EEEEEED576-581QQQQQN reduced cell death to 62 % (P < 0.01).

3.3.5 PHARMACOLOGICAL PROPERTIES OF HETEROMERIC RECEPTORS FOLLOWING CO-TRANSFECTION OF THE MUTANT NMDAR1-1a AND THE WILD-TYPE NMDAR2A

The mutant pCISNMDAR1-1a subunits were co-transfected with the wild type pCISNMDAR2A into HEK 293 cells. Cells were harvested 20 h post-transfection and cell homogenates were subjected to the chemical modification by the treatment with EDAC. The mutants E603Q, EED579-581QQN, EEEEEED576-581QQQQN were all sensitive to the EDAC treatment to the same extent as the wild-type NMDAR1-1a/NMDAR2A receptors and this modification was protected by preincubation with Mg^{2+} . The number of binding sites present in the membranes of HEK 293 cells following co-transfection experiments were similar for all mutant heteromeric receptors to the wild-type NMDAR1-1a/NMDAR2A receptors (not shown). The full pharmacological profile for the mutant NMDAR1-1a(N598Q) co-expressed with NMDAR2A was described, Table 3.3.2. The affinity of mutant heteromeric receptors, NMDAR1-1a (N598Q)/NMDAR2A, for radioligand [3H]MK801 was lower than the affinity of wild-type heteromeric receptors but with a similar number of binding sites. The apparent sensitivity of the same mutant heteromeric receptors to the non-competitive antagonists TCP and ketamine and to divalent cations Mg^{2+} and Ca^{2+} was reduced. No difference in apparent sensitivity to the divalent cation, Zn^{2+} , was observed.

Transfection sample	LDH release (%)			EDAC modification
	unprotected	protected		
		AP5 (200 μ M)	AP5+DKA (200 μ M) each	
NMDAR1-1a	0.0 \pm 0.2			
NMDAR1-1a (N598Q)	0.6 \pm 1.0			
NMDAR2A	0.2 \pm 0.8			
NMDAR1-1a/NMDAR2A	24.5 \pm 2.3	9.3 \pm 1.4	0.0 \pm 0.5	+
NMDAR1-1a (N598Q)/NMDAR2A	12.2 \pm 2.8**	3.4 \pm 1.3	0.1 \pm 0.6	+
NMDAR1-1a (E603V)/NMDAR2A	11.0 \pm 1.7***	0.0 \pm 0.0		+
NMDAR1-1a (EED579 - 581QQN)/NMDAR2A	19.9 \pm 1.3*	2.3 \pm 2.7		+
NMDAR1-1a (EEEEED576- 581QQQQN)/NMDAR2A	15.1 \pm 1.5**	0.8 \pm 0.8		+

Table 3.3.1 **The effect of mutations of the NMDAR1-1a subunit on HEK 293 cell survival and EDAC modification following cotransfection with wild-type and mutant pCISNMDAR1-1a and NMDAR2A.**

HEK 293 cells were transfected with the wild-type and mutant pCISNMDAR1-1a and the pCISNMDAR2A alone or in combination and cultured for 20 h in the presence or absence of the NMDA receptor antagonists. Cell viability was determined by the CytoTox 96TM assay for at least 3 separate experiments as described in 2.2.20. Values are the means \pm S.D. Results were analysed using a two-tailed Student's t-test. *P < 0.02, **P < 0.01, ***P < 0.001, compared to NMDAR1-1a/NMDAR2A wild type.

Sample	³ H]MK801		TCP (nM)	Ketamine (nM)	Mg ²⁺ (mM)	Ca ²⁺ (mM)	Zn ²⁺ (mM)
	K _D (nM)	B _{max} (fmol/mg)					
Mouse forebrain	5.4 ± 0.2	1820 ± 192	75 ± 30	1700 ± 200	0.9 ± 0.1	25.5 ± 7.6	0.33 ± 0.05
NMDAR1-1a /NMDAR2A	6.1 ± 1.4	976 ± 289	67 ± 32 (6)	2600 ± 500	19 ± 7 (6)	121 ± 24	0.53 ± 0.01
NMDAR1-1a (N598Q) /NMDAR2A	38 ± 12	1370 ± 351	3050 ± 770 (4)	70800 ± 20500	0.4 ± 0.2	0.6 ± 0.2	0.58 ± 0.30

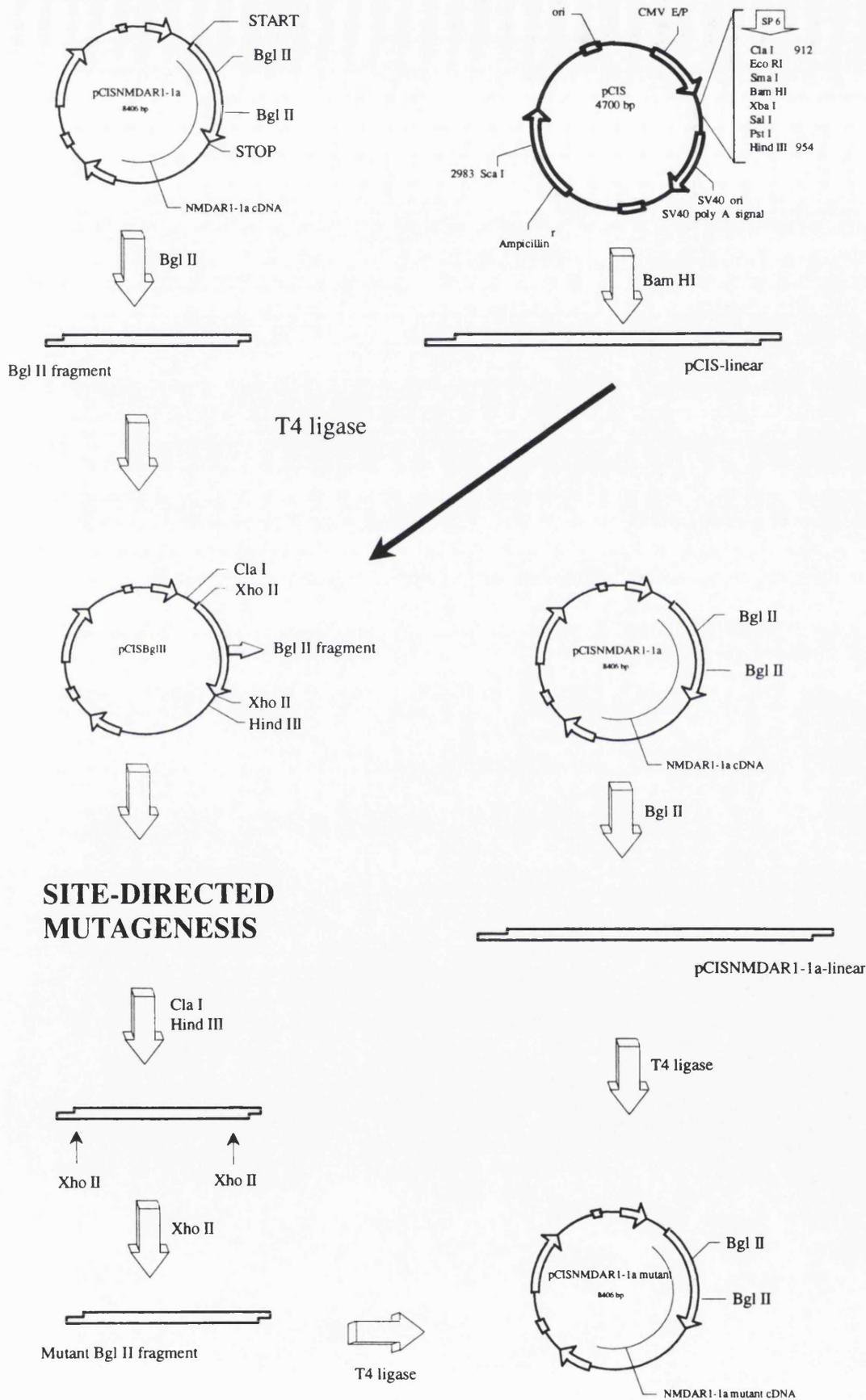
Table 3.3.2 Comparison of the properties of [³H]MK801 specific binding to natural, recombinant and mutant receptors

[³H]MK801 radioligand binding experiments were performed on cell homogenate generated from HEK 293 cells expressing the NMDAR1-1a and NMDAR2A subunits under optimum conditions.

Values are K_I (Ketamine, TCP) or IC₅₀ determinants derived from competition curves, n ≥ 3.

Figure 3.3.1 Strategy for the oligonucleotide-mediated mutagenesis of the NMDAR1-1a subunit.

The *BglIII/BglIII* was subcloned from the pCISNMDAR1-1a into the pCIS and site-directed mutagenesis was performed. Following mutagenesis, the mutated *BglIII/BglIII* fragments were rescued and cloned back into the pCISNMDAR1-1a.



**SITE-DIRECTED
MUTAGENESIS**

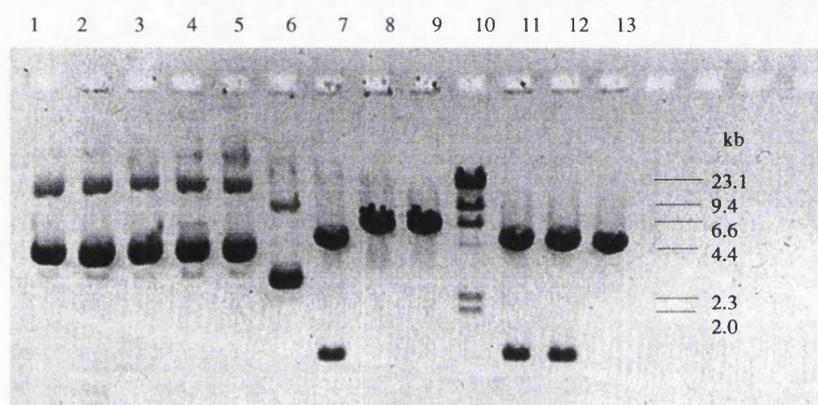


Figure 3.3.2 Restriction enzyme analysis of the recombinant pCISNMDAR1BgIII.

Lanes 1-5	Recombinant pCISNMDAR1BgIII uncut
Lane 6	pCISNMDAR1-1a uncut
Lanes 8 and 9	Recombinant pCISNMDAR1BgIII cut with <i>Eco RI</i> (<i>BglII/BglII</i> fragment in sense orientation)
Lane 10	Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb).
Lanes 7,11 and 12	Recombinant pCISNMDAR1BgIII cut with <i>Eco RI</i> (<i>BglII/BglII</i> fragment in antisense orientation)
Lane 13	pCISNMDAR1-1a cut with <i>Eco RI</i>

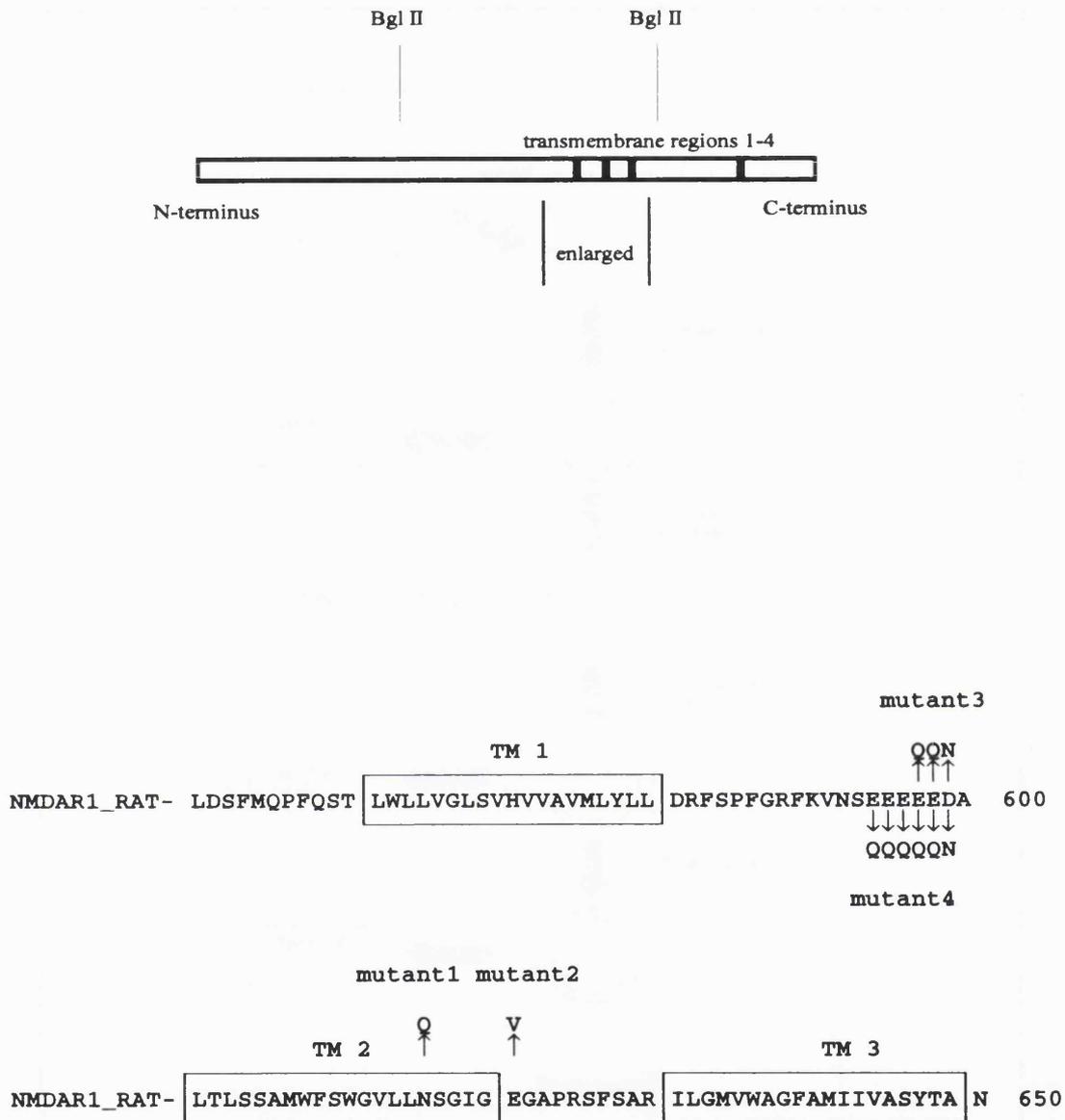


Figure 3.3.3 Schematic representation of the structure of the NMDAR1 subunit showing positions of mutations.

Figure 3.3.3 The schematic representation of the NMDAR1 subunit, showing positions of mutations.

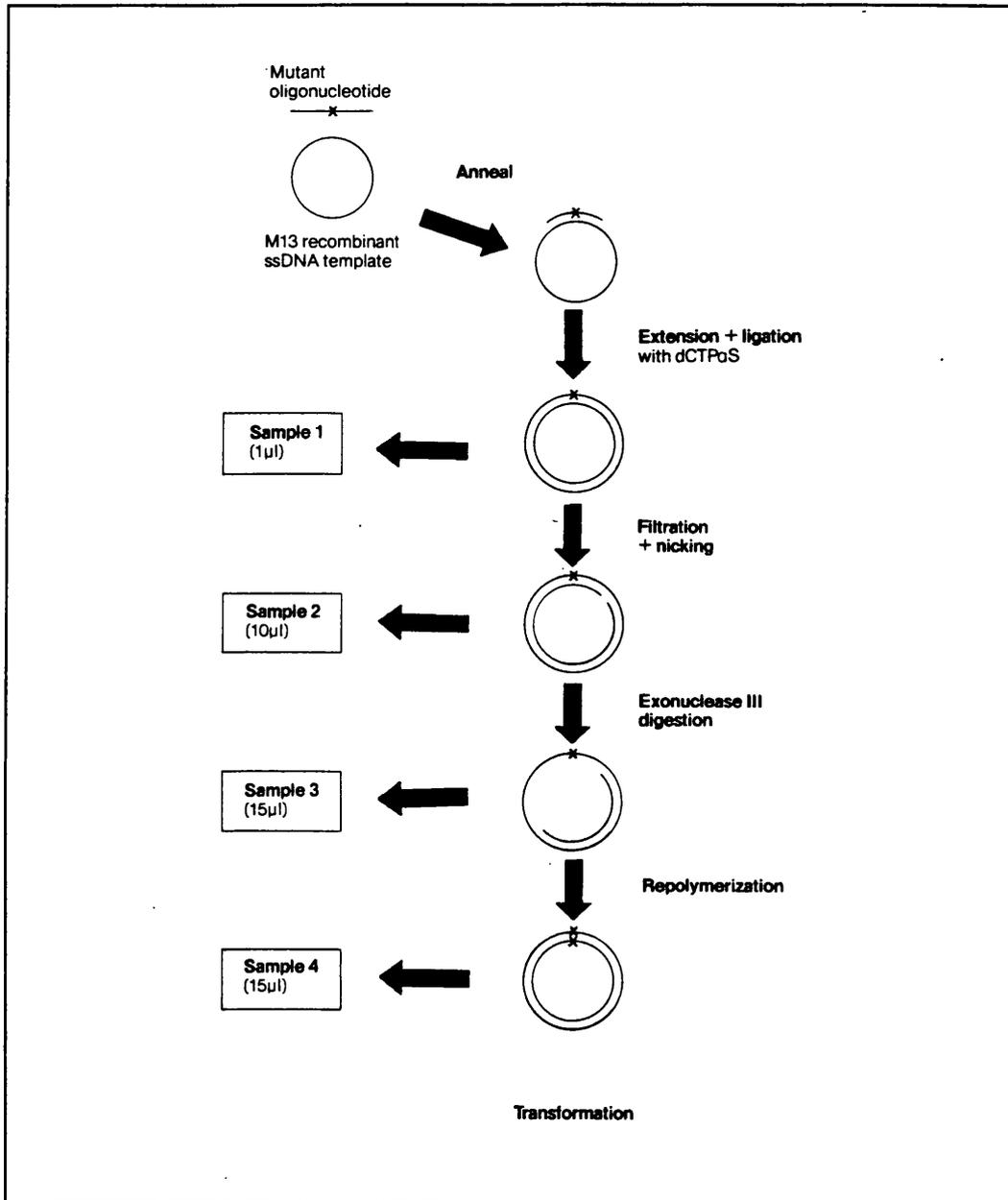


Figure 3.3.4 Schematic representation of the analysis of the *in vitro* mutagenesis reaction.

(From Oligonucleotide-directed *in vitro* mutagenesis system, version 2.0, Amersham International plc)

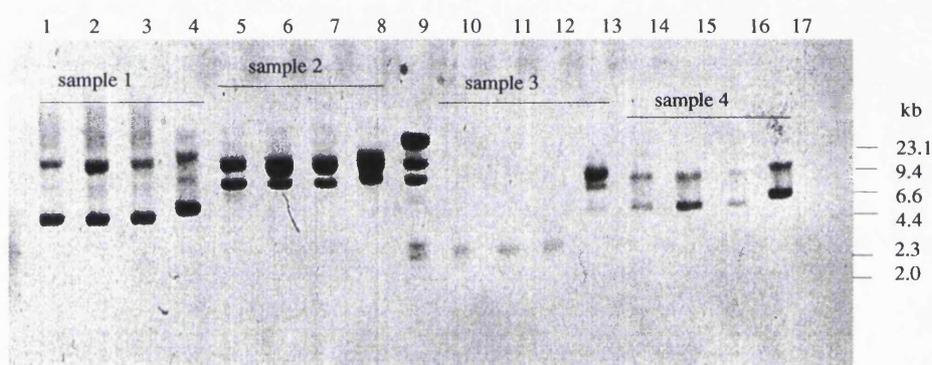


Figure 3.3.5 Analysis of the *in vitro* mutagenesis reaction.

Each sample was loaded in the following order: E598V mutant, EED579-581QQN mutant, EEEEEED576-581QQQQQN mutant, control mutant (M13 mp8 vector). Sample 1 shows the presence of covalently closed circular DNA with smear of nicked DNA above it. Sample 2 shows increase in amount of nicked DNA. Sample 3 shows DNA after Exonuclease III digestion, difference in lanes 10-12 and 13 are due to different template vectors (pCIS versus M13). Sample 4 shows DNA after the final repolymerization step. Lane 9, Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb).

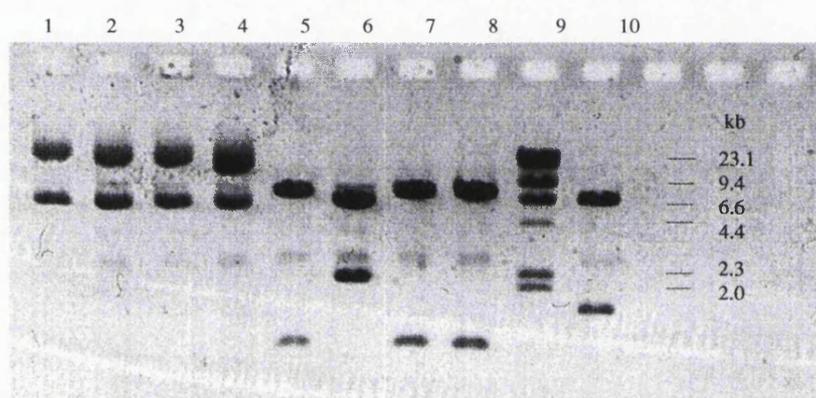


Figure 3.3.6 Analysis of the subcloning of the mutated *BglII/BglII* fragment into the pCISNMDAR1-1a.

- | | |
|--------------|---|
| Lanes 1-4 | pCISNMDAR1-1a (N598Q) uncut |
| Lanes 5, 7-8 | pCISNMDAR1-1a (N598Q) cut with <i>Eco RI</i>
(fragment in sense orientation, sizes 5.3 and 1.1 kb) |
| Lane 6 | pCISNMDAR1-1a (N598Q) cut with <i>Eco RI</i>
(fragment in antisense orientation, sizes 4.3 and 2.1 kb) |
| Lane 9 | Standard MW markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 kb) |
| Lane 10 | pCISNMDAR1-1a (N598Q) cut with <i>Bgl II</i>
(sizes 4.7 and 1.6 kb) |

3.4 DISCUSSION

Site-directed *in vitro* mutagenesis has shown that the Ca^{2+} permeability of the GluR2 subunit of the AMPA-selective glutamate receptor is determined by the presence of arginine at position 586 in the putative TM2 region (Verdoorn *et al.*, 1991). The corresponding positions of the NMDA receptor subunits (NMDAR1, R2A-R2D), are occupied by asparagine (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992). This position within the TM2 of the NMDAR1 subunit (N598), has been changed by site-directed mutagenesis and electrophysiological properties of the mutant studied following expression in the *Xenopus* oocytes and HEK 293 cells (Mori *et al.*, 1992; Burnashev *et al.*, 1992b; Sakurada *et al.*, 1993; Kawajiri and Dingledine, 1993; Ruppertsberg *et al.*, 1993). It has been demonstrated that this site is responsible for control of Ca^{2+} permeability and Mg^{2+} blockade of the NMDA receptor (e.g. Burnashev *et al.*, 1992b). When asparagine was changed to glutamine (N598Q), and the mutant NMDAR1-1a(N598Q) co-expressed with the NMDAR2A in *Xenopus* oocytes, reduced Ca^{2+} permeability and a slight reduction of Mg^{2+} block was observed. External Ca^{2+} acts as a weak channel blocker. At a concentration of 1.8 mM, Ca^{2+} reduced the inward current to 77 %, but the mutation (N598Q) increased the blocking effect of inward currents to 45 % of wild-type receptor (Burnashev *et al.*, 1992b). Extended studies on the single channel properties of the heteromeric NMDAR1-1a(N598Q) / NMDAR2A receptor, showed similar Ca^{2+} blockade of inward current to 40 % of control (Ruppertsberg *et al.*, 1993). When the homologous asparagine (N595Q) was mutated in the NMDAR2A subunit, Ca^{2+} permeability was not significantly changed when heteromeric NMDAR1-1a / NMDAR2A(N595Q) receptors were expressed (Burnashev *et al.*, 1992b). When asparagine was replaced by arginine (N598R), and the heteromeric NMDAR1-1a(N598R) / NMDAR2A receptors were expressed in oocytes, no Ca^{2+}

permeability and no Mg^{2+} blockade could be measured.

The putative TM2 region of the NMDAR1 subunit and amino acids adjacent to that region, have been subjected to extensive site-directed mutagenesis (e.g. Sakurada *et al.*, 1993; Kawajiri and Dingledine, 1993). In one of these mutations, a glutamate was replaced by glutamine (E603Q) and effects of this mutation were studied in the *Xenopus* oocytes (Sakurada *et al.*, 1993; Kawajiri and Dingledine, 1993). This mutation did not have any effect on response to application of NMDA (100 μ M) and glycine (10 μ M), on Mg^{2+} block or MK801 block of homomeric NMDAR1-1a (E603Q) receptor. When heteromeric NMDAR1-1a (E603Q) / NMDAR2A receptors were expressed in oocytes, no changes in Ba^{2+} permeability was observed (Kawajiri and Dingledine, 1993). The stretch of 6 acidic residues, (576EEEEED581), at the amino-terminal side of the TM2 segment of the NMDAR1 was also characterised by site-directed mutagenesis (Mori *et al.*, 1992; Sakurada *et al.*, 1993). Substitution of this stretch by the corresponding amino-acid residues of the GluR1 subunit of the AMPA receptor did not have an effect on Mg^{2+} block, MK801 inhibition or Zn^{2+} inhibition of heteromeric NMDAR1-1a(EEEEEED576-581TSDQSN) / NMDAR2A receptors expressed in *Xenopus* oocytes. When each of these residues was changed to its amide derivative and the properties of each single substitution examined upon homomeric expression in *Xenopus* oocytes, no significant effect on current amplitude, Mg^{2+} block or MK801 inhibition was observed.

In this study, a selection of mutations were chosen to be introduced into the NMDAR1-1a subunit. These mutations were N598Q, E603A, E603V, EED579-581NNQ and EEEEEED579-581NNNNQ. The rationale for the N598Q mutation was that this mutation reduced the Ca^{2+} permeability of the NMDA receptor, as described above. Thus this mutation was chosen to test our hypothesis that following expression of the heteromeric NMDAR1-1a/NMDAR2A receptors, Ca^{2+} influx through NMDA receptor channels is

responsible for cell death. The rationale for mutations of acidic amino acids, (E603 and 576EEEEED581), was that it has been reported in this laboratory that a carboxyl group is important for the binding of [³H]MK801 to native membrane-bound and recombinant heteromeric NMDA receptors (Chazot *et al.*, 1993; Chazot *et al.*, 1994a). Chemical modification of carboxyl groups by treatment with EDAC, resulted in the inhibition of specific [³H]MK801 activity. This inhibition was protected by preincubation with Mg²⁺. In this study, the acidic amino acids adjacent to the putative TM2, were mutated to their amide derivatives.

A part of the NMDAR1-1a sequence (Figure 3.3.2) was subcloned into the pCIS vector to reduce the size of DNA subjected to the mutagenesis reaction (3.3.1). The mutagenesis reaction was performed as described in 3.2.4.1-8, with mutant oligonucleotides and a control oligonucleotide. The control mutagenesis reactions showed 79 % and 95 % mutation efficiency in two separate experiments respectively. The samples, taken from each step of the mutagenesis reaction and analysed on agarose gel (Figures 3.3.3-4), were also indicative of a successful experiment. The putative mutant colonies were analysed by dideoxy sequencing using ss and ds sequencing primers (3.2.4.1). The mutants (N598Q, E603Q, EED579-581QQN and EEEEEED576- 581QQQQQN), were sequenced through the mutated region and only the specific changes were confirmed to be introduced into the coding region. The putative mutant (E603A) was also sequenced through mutated region (8 colonies) but in none of them was a specific mutation observed. The mutant oligonucleotide (E603A) did not satisfy the criteria for the efficient priming to the template DNA, because it had a potential to form a stem-loop and therefore to self anneal. This accounted for the lack of success in eliciting the specific point mutation using that primer. Following the site-directed mutagenesis reaction, the mutated *Bgl II* fragments were subcloned back into the pCISNMDAR1-1a recombinant (3.3.3), generating

the following mutant recombinants; the pCISNMDAR1-1a(N598Q), the pCISNMDAR1-1a (E603V), the pCISNMDAR1-1a(EED579-581QQN) and the pCISNMDAR1-1a (EEEEED576-581QQQQN).

The expression of the mutant NMDAR1-1a polypeptides was verified by immunoblotting using anti-NMDAR1-1a antibodies (results not shown). The number of expressed receptors following the co-expression of the mutant NMDAR1-1a and the wild-type NMDAR2A subunits was determined by [³H]MK801 radioligand binding assays and compared to wild-type NMDAR1-1a / NMDAR2A receptors. There were no significant differences between B_{max} values of mutant heteromeric receptors and wild-type heteromeric NMDA receptors (3.3.5).

When heteromeric NMDAR1(N598Q) / NMDAR2A receptors were expressed in HEK 293 cells, it was observed that the mutation NMDAR1(N598Q) decreased receptor-mediated cell death (Table 3.3.1, Cik *et al.*, 1994). This result demonstrated the validity of our hypothesis that the Ca²⁺ influx, following the activation of expressed heteromeric NMDA receptors is responsible for cell death. The concentration of Ca²⁺ in the culture medium was 1 mM, but the effective concentration was probably higher due to the presence of Ca²⁺ in FCS and due to a contribution of Ca²⁺ during the transfection procedure. Thus, the concentration of Ca²⁺ in the culture medium was high enough to exert Ca²⁺ blockade of the mutant NMDA receptor channel, subsequently decreasing the Ca²⁺ influx into HEK 293 cells and decreasing cell mortality. Both Ca²⁺ blockade and reduced Ca²⁺ permeability were described for heteromeric NMDAR1(N598Q) / NMDAR2A receptors (Burnashev *et al.*, 1992b). Protection against the residual percentage of cell death was also, as for the heteromeric wild-type NMDA receptor, achieved by the presence of AP5 in the culture medium, or optimally by the presence of both AP5 and DKA suggesting that mutation had no major effect on agonist binding sites (Table

3.3.1). Similar results following expression of NMDAR1-1a(N598Q) / NMDAR2A were described by Anegawa *et al.*, (1993), except that in that preliminary report, the presence of AP5 or DKA in medium post-transfection had no effect on protection against cell death.

Following expression of heteromeric NMDAR1-1a(E603V) / NMDAR2A receptors in HEK 293 cells significant reduction in cell death was observed. Cell mortality was reduced to 45 % ($P < 0.001$) compared to control HEK cells expressing wild-type receptors and protection against the residual percentage of cell death was achieved by the presence of AP5 in the culture medium post-transfection (Table 3.3.1). The mutation (EED579-581QQN) had a minor effect on HEK 293 cell mortality which was reduced to 81 % ($P < 0.02$). Cell death was more significantly reduced for the (EEEEED576-581QQQQQN) mutant, reducing HEK 293 cell mortality to 62 % ($P < 0.01$) of control cells expressing NMDAR1-1a / NMDAR2A heteromeric receptors (Table 3.3.1). These results suggested that the residues E603 and 576EEEEED581 could be involved in the regulation of Ca^{2+} permeability of heteromeric NMDAR1-1a / NMDAR2A receptors. The mechanism of this regulation remains to be investigated but could result from changes in Ca^{2+} binding, Ca^{2+} -channel blockade, the voltage-dependent Mg^{2+} blockade or agonist and/or co-agonist affinity.

The mutant NMDAR1-1a subunits were co-expressed with the wild-type NMDAR2A subunit into HEK 293 cells. Following co-transfection, the cell homogenates were subjected to EDAC treatment. All of these heteromeric NMDA receptors (mutant NMDAR1-1a / NMDAR2A) were sensitive to the EDAC modification to the same extent as wild-type NMDAR1-1a / NMDAR2A receptors. Moreover, the affinity of Mg^{2+} in the inhibition of [3H]MK801 binding was unchanged compared to wild-type heteromeric receptors demonstrating that these mutated residues were not involved in the Mg^{2+} binding site. Thus, the carboxyl group involved in EDAC modification has not been identified. As it is

possible that this group is present within a channel forming region of the NMDAR2A or within TM1 region, which was also identified to have effect on channel properties on non-NMDA receptors (Köhler et al., 1993), further mutagenesis experiments should be performed.

CHAPTER 4

General discussion and future perspectives

The aim of this project was to establish conditions for the optimal transient expression of homo- and heteromeric NMDA receptors in mammalian cells. Following the cloning of the NMDAR1 subunit (Moriyoshi *et al.*, 1991) and the NMDAR2A-2D subunits (e.g. Meguro *et al.*, 1992), their properties were characterized electrophysiologically in *Xenopus* oocytes and mammalian cells (e.g. Ikeda *et al.*, 1992).

To analyze the cloned NMDA receptors on a biochemical level by using pharmacological and immunological tools in this project expression in mammalian cells was used. It was already reported that HEK 293 cells and the CMV promoter/enhancer based vector could be used for the efficient expression of neurotransmitter ionotropic receptors (e.g. Werner *et al.*, 1991). Thus, the rat NMDAR1-1a and the mouse NMDAR2A subunits were subcloned into the pCIS, mammalian expression vector (Gorman *et al.*, 1990). During the subcloning, an effort was made to reduce the 5' and 3' untranslated regions to improve the transcription efficiency. It was described recently that the presence of the long 5' untranslated region reduces the expression of full length functional NMDA polypeptides in *Xenopus* oocytes (Williams, 1993). As it was difficult to subclone the NMDAR2A cDNA in one step, probably due to its size being larger than pCIS itself, a 2 step subcloning procedure was adopted (2.3.2).

The control vector pSV- β -galactosidase was used for the optimisation of the important parameters in the calcium phosphate transfection procedure. These important parameters included the pH of the media and the cell number at the moment of transfection (2.3.3). Both the NMDAR1-1a and the NMDAR2A subunits were expressed alone in HEK 293 cells as demonstrated immunologically with anti-NMDAR1-1a and anti-NMDAR2A antibodies. It was found that both subunits were N-glycosylated polypeptides with M_r of 117 000 and 180 000 respectively. N-Deglycosylation of these polypeptides resulted in immunoreactive species of M_r 97 000 and 165 000 for NMDAR1-1a and NMDAR2A respectively, which was in good agreement with the predicted

molecular weights for the respective cDNAs. Similar results for the NMDAR1 subunit were published recently (Brose *et al.*, 1993; Sucher *et al.*, 1993; Tingley *et al.*, 1993). [³H]MK801 specific binding activity, albeit very low was detected only in cell homogenates prepared from HEK 293 cells transfected with the NMDAR1-1a suggesting that the MK801 binding site is present within the NMDAR1 subunit. The advent of potentially more selective photoaffinity probes would aid in the localisation of this and other important modulatory binding sites within the NMDA receptor complex (Benke *et al.*, 1993).

Co-transfection of the NMDAR1-1a and the NMDAR2A subunits resulted in cell death (3.3.5). It was determined by co-expression of the control vector pSV- β -galactosidase with the NMDAR1-1a and the NMDAR2A subunits, that all co-transfected cells died (2.2.5.3). As under the standard transfection conditions cells were grown in the presence of glutamate and glycine in the FCS containing medium, it was proposed that glutamate activation of heteromeric NMDA receptors led to Ca²⁺ influx into cells and subsequently to cell death. Further experiments should be performed to assess the dose-dependence of cell death to glutamate and glycine in a serum-free medium. Also, experiments should be performed to measure more directly Ca²⁺ influx in co-transfected cells. This could be achieved by measuring the influx of ⁴⁵Ca²⁺ (e.g. Janáky *et al.*, 1993) or by fura-2 Ca²⁺ imaging (e.g. Tymianski *et al.*, 1993).

In this thesis experiments were performed only with the two NMDA receptor subunits, NMDAR1-1a and NMDAR2A, further experiments should be performed to address involvement of the other NMDA subunits, NMDAR2B-2D, and the other NMDAR1 splice variants in the structure-function relationship and receptor-mediated cell death. It has been reported by others that different combinations of the NMDAR1 and the NMDAR2A subunits had different sensitivity to agonists, antagonists and Mg²⁺ blockade (e.g. Ikeda *et al.*, 1992). Preliminary results with the NMDAR2C subunit expressed together with the NMDAR1-1a or with the NMDAR1-1a and NMDAR2A subunits showed no cell

death for the NMDAR1-1a/NMDAR2C combination and differential effects on cell death depending on the DNA ratio upon triple co-transfection. There is also preliminary evidence that the different DNA ratios used for triple co-transfection results in different pharmacological properties of expressed heteromeric NMDA receptors.

To prevent cell death co-transfected cells were grown in the presence of the NMDA receptor antagonist AP5 in glutamine-free culture medium post-transfection. It was reported that a glutamine-free medium significantly increased cell viability of primary neurons in culture (Driscoll *et al.*, 1993). Furthermore, following expression of heteromeric NMDA receptors in *Xenopus* oocytes, other laboratories have used AP5 to prevent the death of *Xenopus* oocytes (e.g. P. Stern personal communication). The prevention of cell death was further investigated by the inclusion of different NMDA receptor antagonists alone or in pair-wise combination (2.3.5.2). The effect of these antagonists on cell death was measured by trypan blue exclusion and the CytoTox 96TM assay. Only the presence of both AP5 and DKA in the cell culture medium post-transfection resulted in complete prevention of cell death.

The time-dependent appearance of immunoreactivity and [³H]MK801 specific binding activity was followed for up to 62 h post-transfection (2.3.5.1.1). The maximum [³H]MK801 specific binding activity was observed at 24 h post-transfection, which was concomitant with the maximum immunoreactivities observed. Both [³H]MK801 specific binding activity and immunoreactivity remained at approximately the same level for up to 62 h post-transfection. The maximum [³H]MK801 specific binding activity was 10-fold larger than [³H]MK801 specific binding activity measured following expression of the NMDAR1-1a subunit alone (2.3.3). Single-channel recordings in an outside-out patch were performed at the time point 20 h post-transfection (2.3.5.3). Single channel properties were very similar to single channels recorded in oocytes expressing the same subunit combination, suggesting that there was no difference

between the two expression systems and between the rat and mouse NMDAR1-1a/NMDAR2A receptors. Moreover these properties were similar to single channels recorded in the hippocampal neurons and cerebellar granule cells in culture (Stern *et al.*, 1992; Stern *et al.*, 1994a; Stern *et al.*, 1994b). At the present time little is known about the subunit composition of native NMDA receptor-channels. The candidate native heteromeric NMDAR1-1a/NMDAR2A receptors had electrophysiological properties very similar to hippocampal CA1 neurons and to cerebellar granule cells (Stern *et al.*, 1992; Stern *et al.*, 1994b). Moreover *in situ* hybridization demonstrated the presence of both NMDAR1-1a and NMDAR2A mRNAs in these regions (Ishii *et al.*, 1993). Thus results presented in this thesis may be relevant for these subpopulations of native NMDA receptors.

These results taken together demonstrate that an efficient system for transient expression of the NMDA receptor subunits was established. The observed cell death, as result of the expression of heteromeric NMDA receptors opens up possibilities for new insights into the NMDA receptor-mediated mechanisms that underline cell death. One of the approaches to study these mechanisms is site-directed mutagenesis, which was used to generate mutations within and adjacent to the TM2 region of the NMDAR1-1a subunit (3.3.1). It was already demonstrated by site-directed mutagenesis that amino acid N598 within the TM2 is responsible for control of Ca^{2+} permeability and Mg^{2+} blockade of the NMDA receptor (Burnashev *et al.*, 1992b). When the mutant NMDAR1-1a(N598Q) was co-expressed with the NMDAR2A subunit, a significant decrease in cell death was observed, which was in good agreement with the hypothesis that receptor-mediated Ca^{2+} influx in transfected cells was responsible for the observed cell death (3.3.4).

In this study, other mutations within the region adjacent to TM2 of the NMDAR1-1a were also tested for their effect on cell death. Although it was reported in electrophysiological studies that the mutation E603Q appeared not to

have any effect on Ba^{2+} permeability (Kawajiri and Dingledine, 1993), in this study it was found that the mutation NMDAR1-1a(E603V) significantly reduced cell death following co-transfection with the NMDAR2A, suggesting an involvement of this residue in the regulation of Ca^{2+} permeability (3.3.4). It has been reported that different substitutions of the same residue had different effects on receptor properties (e.g. Bertrand *et al.*, 1993; Kawajiri and Dingledine, 1993). Therefore, limited mutagenesis experiments should be viewed with a degree of caution. Thus, the mutation of E603 residue to a range of amino acids with different side chains should be performed. The two mutations of the acidic amino acids adjacent to the TM2 region, 576EEEEED581, had differential effect on the reduction of cell death which also suggested their involvement in the regulation of Ca^{2+} permeability but further mutagenesis experiments should be performed to confirm both of these observations.

The establishment of a model system for the studying of the NMDA receptor properties at the molecular level has opened up many possibilities for the elucidation of the structural determinants underlying native NMDA receptor heterogeneity. Furthermore, this model system may aid in the identifying of mechanisms underlying the receptor-mediated cell death. Finally, it would be beneficial to investigate the possibility of studying expression of this and other putative native receptors in neuronal cell lines in order to determine the intracellular pathway or pathways leading to "neuronal" cell death. As always in science this is just a beginning.

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- Chazot, P.L., Cik, M. and Stephenson, F.A. (1992) Immunological detection of the NMDAR1 glutamate receptor subunit expressed in Human Embryonic Kidney 293 cells and in rat brain. Journal of Neurochemistry **59** (3) 1176-1178
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IMMUNOLOGICAL CHARACTERISATION OF THE NMDAR1 GLUTAMATE RECEPTOR IN TRANSFECTED MAMMALIAN CELLS AND RAT BRAIN

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A complementary DNA (cDNA) encoding the rat NMDA glutamate receptor (NMDAR1) was recently cloned by functional expression in the *Xenopus* oocyte¹. In this present study, we have transiently expressed the NMDAR1 into a mammalian cell line with a view to the production of a simple model system for the study of structure-activity relationships. Thus, the NMDAR1 clone was excised from the pN60 plasmid¹ and subcloned into the *SmaI/XbaI* sites of the pCIS expression vector². Human embryonic kidney cells were transfected using the calcium phosphate precipitation procedure², and harvested after 48 hours. Recombinantly expressed NMDA receptors were characterised with respect to both their ligand binding and immunological properties. [³H] MK801 in the presence of 10 μ M glutamate bound specifically to the membranes prepared from the transfected cells; binding activity was 102 ± 20 fmoles/mg protein (n=5) which corresponded to ~ 40000 receptor sites/cell.

Polyclonal antibodies were raised in rabbits to a synthetic peptide corresponding to the C-terminal amino acid sequence of the NMDAR1 receptor¹ conjugated to keyhole limpet haemocyanin³. A high titre antiserum was generated (>1:50000 with the peptide as antigen), and the antibody was purified by respective peptide-affinity chromatography³. Purified anti-NMDAR1 antibodies were used to probe rat brain and transfected 293 cell membranes by Western blotting analysis³. A single immunoreactive species was detected in rat brain membranes (M_r 117000 \pm 2000, n=5). In contrast, two species of M_r 117000 \pm 2000 and M_r 97000 \pm 1000 (n=5) were detected in transfected cell membranes, neither of which were observed in untransfected 293 cell membranes in parallel analysis. The immunoreactive species were all suppressed by prior incubation with peptide (500 μ g/ml). The identity of the lower molecular weight band may be a proteolytic fragment or a differentially glycosylated form of the receptor. The assignment of the M_r 117000 immunoreactive species as the NMDAR1 receptor polypeptide is consistent with the predicted molecular size from the deduced amino acid sequence (M_r 105000) allowing for a contribution by weight for N-glycosylation¹, and with a previous report using [³H] azido MK801 as a photoaffinity label in rat brain⁴.

This work was funded by the SERC (UK). The authors wish to thank K. Moriyoshi and coworkers (Kyoto, Japan) for the generous gift of the NMDAR1 clone.

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AN ANTI-NMDAR2A GLUTAMATE RECEPTOR SUBUNIT-SPECIFIC ANTIBODY: USE IN THE CHARACTERISATION OF NATIVE AND RECOMBINANT RECEPTOR HETERO-OLIGOMERS. Paul L Chazot, Miroslav Cik and F Anne Stephenson, School of Pharmacy, 29-39 Brunswick Square, London WC1N 1AX, UK.

Molecular cloning has identified five genes encoding the N-methyl-D-aspartate (NMDA) glutamate receptor subunits, NMDAR1 and NMDAR2A(1). Coexpression of different combinations of these suggest that native NMDA receptors are hetero-oligomeric. In order to investigate this at the protein level, we have adopted an immunological approach. We have previously reported the identification of the NMDAR1 subunit, M_r 117000, in rat brain and in mammalian cells transfected with pCISNMDAR1 using an anti-NMDAR1 929-938 antibody(2). Now, we report the production of an anti-NMDAR2A subunit-specific antibody and its use in the characterisation of native and recombinant NMDAR1/NMDAR2A subunit-containing glutamate receptors. Thus, affinity-purified anti-NMDAR2A 1435-1445 antibodies specifically labelled four species with M_r 180000, 122000, 67000 and 54000, in membranes prepared from adult rat forebrain. In cells transfected with pCISNMDAR2A alone, these antibodies recognised a single immunoreactive species with M_r 180000. N-Deglycosylation yielded one band with M_r 165000 consistent with the predicted molecular weight of the NMDAR2A polypeptide. Coexpression of NMDAR1/NMDAR2A resulted in an 8-fold increase in [³H] MK801 specific binding activity compared to either of the singly expressed subunits. This finding further substantiates the coassociation of these two subunit types in agreement with both electrophysiological characterisation of heterologous receptors expressed in oocytes and *in situ* hybridisation studies. Further investigations of native and cloned NMDA receptors, in parallel, using the above-described biochemical and immunological approaches should provide additional insights into NMDA receptor structure and function. We thank Drs Nakanishi and Mishina for the generous gifts of cDNA. Funded by SERC (UK).
(1) Nakanishi, S (1992) Science 258, 597-603.
(2) Chazot, P.L., Cik, M. and Stephenson, F.A. (1992) J. Neurochem. 59, 1176-1178.

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OPTIMISATION OF THE EXPERIMENTAL CONDITIONS FOR THE MAXIMAL EXPRESSION OF NMDAR1/NMDAR2A HETEROMERIC RECEPTORS IN MAMMALIAN CELLS. Paul L. Chazot, Miroslav Cik* and F. Anne Stephenson, School of Pharmacy, 29/39 Brunswick Square, London, WC1N 1AX, UK.

Several cDNAs encoding the rat NMDA subclass of glutamate receptors have recently been identified, namely the NMDAR1 and NMDAR2A-2D subunits (1). We have transiently expressed the NMDAR1 and NMDAR2A either singly or in combination in human embryonic kidney 293 cells. Receptor expression was monitored by both [³H] MK801 radioligand binding assays and immunoblotting using anti-NMDAR1 and NMDAR2A subunit-specific antibodies (2,3). Cotransfection with NMDAR1/NMDAR2A resulted in cell death which was prevented by the inclusion of 2-amino-5-phosphonopentanoic acid (AP5) in the culture medium immediately following transfection. Under these conditions, both the time course for the expression of heteromeric receptors and the ratio of the respective recombinant plasmids used for transfection were investigated. Optimal expression was achieved with a 1:3 ratio of pCISNMDAR1/NMDAR2A. Immunoreactivity was detectable 3h post-transfection; at 15h [³H] MK801 binding activity was at a maximum and ~ 10 fold higher than found for single subunit expression. At 24h, overexpression as defined by the detection of non-N-glycosylated proteins occurred and cell division was attenuated. However, [³H] MK801 binding activity and respective immunoreactivities remained stable for a further 48h. Thus we have optimised the experimental conditions for the maximal expression of heteromeric NMDA receptors which permits their biochemical characterisation. This provides a useful model for the study of the regulation of NMDA receptor assembly.

We thanks Drs Nakanishi and Mishina for the generous gifts of cDNAs. Funded by SERC (UK).

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TRANSIENT EXPRESSION OF NMDAR1/NMDAR2A HETEROMERIC RECEPTORS IN MAMMALIAN CELLS: CELL VIABILITY AS AN INDEX OF EFFICIENT FUNCTIONAL ASSEMBLY

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L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. Within the ionotropic glutamate receptors, two pharmacological subclasses were defined which were those activated by NMDA, i.e. NMDA receptors and those insensitive to NMDA, i.e. the non-NMDA receptors. Molecular cloning has identified five genes encoding NMDA glutamate receptor subunits, NMDAR1 and NMDAR2A-D (1). We have transiently expressed the N-methyl-D-aspartateR1 (NMDAR1) and NMDAR2A subunits either alone or in combination in human embryonic kidney (HEK) 293 cells (2,3). The biochemical and pharmacological properties of the cloned receptors were compared with those of adult, rat brain NMDA receptors using both immunological methods with a newly-developed anti-NMDAR2A (1435-1445) antibody and [³H] MK801 radioligand binding activity. Anti-NMDAR2A antibodies recognised specifically four immunoreactive species with M_r s 180 000, 122 000, 97 000 and 54 000 in rat brain but a single band with M_r 180 000 in HEK 293 cells singly transfected with pCISNMDAR2A. N-Deglycosylation of HEK 293 cell membranes yielded a M_r 165 000 immunoreactive species which is in agreement with the size predicted from the cDNA sequence for the mature NMDAR2A subunit. Coexpression of NMDAR1 and NMDAR2A subunits in HEK 293 cells resulted in cell death. Thus conditions were established for the optimum expression of heteromeric receptors in viable cells which included a requirement for DL-2-amino-5-phosphonopentanoic acid (AP5) in the culture medium post-transfection. Although the cotransfected HEK 293 cells were grown in the presence of AP5 (200 μ M), some cell death was apparent compared to non-transfected or singly transfected HEK 293 cells. The NMDA receptors are subject to multiple allosteric modulation by a strychnine-insensitive glycine binding site, polyamines, Mg^{2+} and Zn^{2+} cations. We have investigated the effect of some of these agents, all known to antagonise NMDA receptor activation, on the recombinant NMDAR1/NMDAR2A receptors. The cotransfected HEK 293 cells were grown in the presence of one, or a combination of two agents and their effect was monitored by trypan blue exclusion. The results are summarised in Figure 1. The singly transfected HEK 293 cells (NMDAR1 or NMDAR2A) had the same number of non-viable cells compared to nontransfected cells. The combination of AP5 and the antagonist of the strychnine-insensitive glycine site, 5,7-dichlorokynurenic acid (DKA), gave optimal results with no significant difference between the percentage of cell death in control and test samples. It was interesting that even DKA alone partially protected against cell death. The integrity of the NMDAR1 and NMDAR2A subunits was maintained, as determined by immunoblotting under all these protection conditions. The non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), had no effect on cell death. Cells transfected with

pCISNMDAR1 and pCISNMDAR2A combined yielded a ten-fold increase in the number of [³H] MK801 binding sites compared to single subunit expression. MK801 had similar affinity for the expressed receptors to those of adult rat and mouse brain. These results demonstrate that the NMDAR1 and NMDAR2A receptor subunits coassemble to form a heteromeric complex with properties similar to native receptors of adult mammalian forebrain. Furthermore, the conditions reported for maximal transient expression provide a basis for further structure-activity studies.

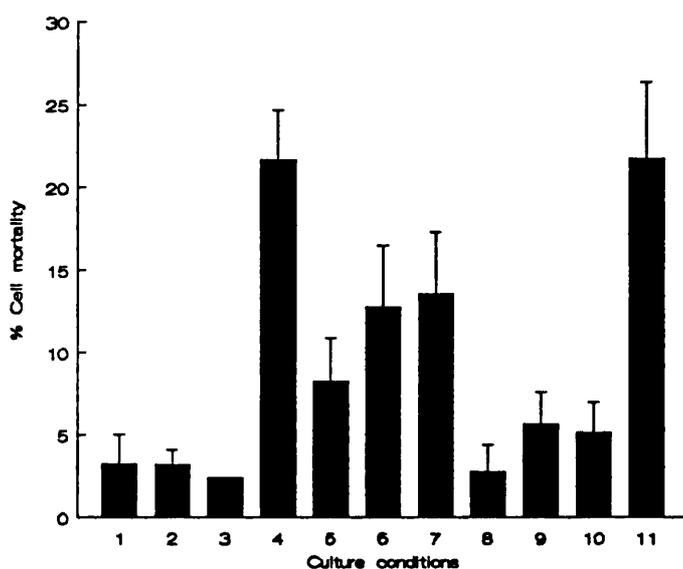


FIGURE 1. The effect of NMDA receptor ligands on cell survival following cotransfection with pCISNMDAR1 and pCISNMDAR2A.

HEK 293 cells were cotransfected with pCISNMDAR1 and pCISNMDAR2A and cultured for 20 h in the presence of different pharmacological agents. Cells were harvested, collected by centrifugation and the percentage of cell viability determined by trypan blue exclusion. Control samples are:- 1, untransfected cells (n = 2); 2, cells transfected with pCISNMDAR1 alone (n = 3); 3, cells transfected with pCISNMDAR2A alone (n = 2). Samples 3-11 are all cotransfections and the culture conditions are:- 4, no additions (n = 11); 5, 2 x AP5 (200 μ M each; n = 5); 6, 2 x DKA (200 μ M each; n = 4); 7, 2 x Mg²⁺ (2 x 10 mM each; n = 4); 8, AP5 + DKA (n = 3); 9, AP5 + Mg²⁺ (n = 4); 10, DKA + Mg²⁺ (n = 3); 11, 2 x CNQX (100 μ M each; n = 3). Values shown are the mean \pm S.D. for 10 fields of view for three independent cell counts for each transfection and for (n) separate transfections. Results were analysed using a two-tailed Student's t-test with a criterion of significance of p < 0.05. In comparison to cells grown in the absence of AP5, the degree of significance for the presence of AP5 alone was p < 0.0001; DKA alone p < 0.0001 and Mg²⁺ alone, p < 0.0002. In comparison to cells grown in the presence of AP5 the degree of significance for AP5 + DKA was p < 0.02.

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Characterisation of a cation binding site of a cloned heteromeric NMDA receptor: Comparison with native receptors

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The N-methyl-D-aspartate (NMDA) subclass of excitatory glutamate receptors are fast-acting ligand-gated cation channels. NMDA receptors are regulated at distinct allosteric sites by several endogenous modulators which include glycine, certain polyamines, Mg^{2+} and Zn^{2+} . They are readily assayed *in vitro* using the high affinity NMDA receptor channel ligand, [3H] MK801. Using chemical modification, we have previously reported the importance of a carboxyl group for the binding of [3H] MK801 to native membrane-bound NMDA receptors of rat forebrain. Thus, treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) of brain membranes resulted in a dose-dependent inhibition of specific [3H] MK801 binding activity [1]. This inhibition was protected by pre-incubation of the membranes with Mg^{2+} ($EC_{50} = 1.3$ mM) thus demonstrating a close association between the MK801 and low affinity, inhibitory Mg^{2+} binding sites [1].

Several post-synaptic NMDA receptor genes encoding the subunits NMDAR1 and NMDAR2A-D have recently been identified. These subunits are thought to coassemble in different combinations to yield functional glutamate-gated channels [2]. *In situ* hybridisation studies suggest the coexistence of an NMDAR1 subunit with either the 2A, 2B or 2C polypeptide [e.g., 3]. We have therefore expressed the candidate natural receptor, the NMDAR1/NMDAR2A combination, transiently in human embryonic kidney (HEK) 293 cells [4]. HEK cells were harvested 24 h post-transfection, homogenates prepared and the properties of the expressed receptor were characterised using methods as described previously [4]. Treatment of the cell homogenates with 5 mM EDAC resulted in the inhibition of [3H] MK801 binding sites. The percentage of sites inactivated was $63 \pm 10\%$ ($n =$

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3) compared to an inactivation of $71 \pm 2\%$ ($n = 3$) for rat forebrain membranes both measured at 5 mM EDAC. This inhibition could be prevented, as for the native system, in a dose-dependent manner by preincubation of HEK cell homogenates with Mg^{2+} . However, the EC_{50} for the transfected cells was 14-fold lower than found for rat brain membranes (Table 1). When the pharmacological specificity of the expressed receptor was characterised using [3H] MK801 it was found that the affinities for the competitive inhibitors of MK801 binding, ketamine and tenocyclidine (TCP), were indistinguishable between the native and cloned receptor but Mg^{2+} had lower affinity for the NMDAR1/NMDAR2A receptor (Table 1). The shift in affinity for Mg^{2+} inhibition of direct binding was comparable with the shift in the EC_{50} for protection of [3H] MK801 binding sites against EDAC inactivation. These results demonstrate that the NMDAR1/NMDAR2A cloned receptor has similar properties to native receptors expressed in adult rat forebrain with respect to both their inactivation by EDAC treatment and the affinities of competitive inhibitors of [3H] MK801 binding. Interestingly however, a difference was found in both systems for Mg^{2+} . Candidate amino acids responsible for this affinity change are currently under further investigation by site-directed mutagenesis.

This work was funded by the Science and Engineering Research Council (UK).

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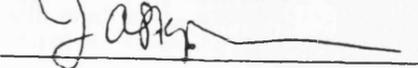
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EXPRESSION OF HETEROMERIC NMDA RECEPTOR SUBTYPES IN MAMMALIAN CELLS RESULTS IN DIFFERENTIAL CELL DEATH
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N-Methyl-D-aspartate (NMDA) receptors have been implicated in mechanisms of neurotoxicity following acute brain damage. This is thought to occur via overstimulation of the NMDA receptor resulting in an increase of intracellular Ca^{2+} . Several genes encoding NMDA receptor subunits NMDAR1 and NMDAR2A-2D have recently been identified (e.g. Meguro et al., 1992). We have studied the effect of the NMDA receptor on cell mortality by the expression of the NMDAR1-1a, NMDAR2A and NMDAR2C either alone or in combination in human embryonic kidney (HEK) 293 cells (Cik et al., 1993). Cell death post-transfection was quantified by either trypan blue exclusion or by the CytoTox 96™ non-radioactive cytotoxicity assay. Receptor expression was monitored by both radioligand binding assay and immunoblotting using subunit-specific antibodies. We found that co-expression of NMDAR1-1a/NMDAR2A subunits resulted in 100 % death of transfected cells (Cik et al., 1994). This observed cell death was prevented by the inclusion of saturating concentrations of the NMDA receptor antagonists either alone or in pair-wise combination in the cell media post-transfection. Further, the percentage cell death was significantly reduced by the expression of NMDAR1-1a(N598Q)/NMDAR2A mutant receptors, a point mutation which is known to reduce the Ca^{2+} permeability of the NMDA receptor channel. In contrast, cells transfected with either NMDAR1-1a, NMDAR2A and NMDAR2C alone or the NMDAR1-1a/NMDAR2C combination despite the same level of receptor subunit expression, resulted in no cell death. These result provide further evidence for the involvement of the NMDA receptor in mechanisms of Ca^{2+} -mediated toxicity. Furthermore, the difference in cell mortality found for the two cloned receptor subtypes suggests differential NMDA receptor channel Ca^{2+} permeability, which has important implications for the development of subtype-selective therapeutic agents.

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Rapid Communication

Immunological Detection of the NMDAR1 Glutamate Receptor Subunit Expressed in Human Embryonic Kidney 293 Cells and in Rat Brain

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Abstract: The rat NMDAR1 (*N*-methyl-D-aspartate receptor) was expressed transiently in human embryonic kidney cells. Transfected cell homogenates showed saturable [³H]MK-801 binding activity that was best fit by a single high-affinity site with a K_D of 9 nM and a B_{max} of 113 fmol of binding sites/mg of protein. Antibodies raised against the peptide sequence NMDAR1 (929–938) coupled to keyhole limpet haemocyanin specifically recognised a single band with M_r 117,000 in immunoblots from adult rat brain. In the transfected cells, the antibody recognised two bands: one with M_r 117,000, which was coincident with that from brain membranes, and one with M_r 97,000, which was identified as nonglycosylated NMDAR1 subunit. These results identify the NMDAR1 of rat brain and further show that the homooligomer binds MK-801, albeit at low efficiency. **Key Words:** *N*-Methyl-D-aspartate—Glutamate—Receptor—Antibody—Glycosylation. **Chazot P. L. et al.** Immunological detection of the NMDAR1 glutamate receptor subunit expressed in human embryonic kidney 293 cells and in rat brain. *J. Neurochem.* **59**, 1176–1178 (1992).

The fast-acting *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptor mediates many of the physiological functions of the excitatory neurotransmitter in the CNS. For example, it is believed to be essential for the induction of long-term potentiation, an activity that may underlie learning and memory (Madison et al., 1991), and it also has the potential to trigger neuronal degeneration and cell death. Intensive study over the last few years has led to a plethora of pharmacological and electrophysiological information relating to the NMDA receptor. Biochemical characterisation of the protein, however, has been minimal owing to both the lack of selective, high-affinity probes and the difficulties in solubilising, with high efficiency, the active receptor. However, Moriyoshi et al. (1991) cloned, by functional expression in the *Xenopus* oocyte, a cDNA encoding a rat NMDA glutamate receptor subunit, NMDAR1. This achievement thus provides important new information with which to initiate the detailed structural characterisation of the NMDA receptor.

In this study, we have transiently expressed the NMDAR1 into a mammalian cell line with a view to the production of a simple model system for the study of NMDA receptor structure–activity relationships. Furthermore, we describe an NMDAR1-selective antibody and its use as an immunological probe to study the structure of both the native and transiently expressed receptor protein.

MATERIALS AND METHODS

Materials

[³H]MK-801 (28.8 Ci/mmol) was purchased from Du Pont (U.K.) Ltd. (Stevenage, Hertfordshire, U.K.). The peptide NMDAR1 (929–938), with an amino acid sequence in single letter code of LQLCSRHRRES, was from Multiple Peptide Systems (San Diego, CA, U.S.A.). Biotinylated anti-rabbit immunoglobulin, streptavidin–peroxidase complex, and enhanced chemiluminescence (ECL) detection system were from Amersham International (Bucks, U.K.). *N*-Glycosidase F was from Boehringer Mannheim GmbH (Lewes, East Sussex, U.K.). pSV- β -Galactosidase was from Promega (Madison, WI, U.S.A.). Human embryonic kidney 293 cells were a gift from Dr. T. G. Smart (School of Pharmacy, London, U.K.), and the pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, CA, U.S.A.). All other materials were from commercial sources.

Plasmid construction and cell transfection

The cDNA encoding the full-length NMDAR1 was excised from the pN60 plasmid (Moriyoshi et al., 1991) and cloned directionally into the *Sma*I/*Xba*I sites of the pCIS mammalian expression vector (Gorman et al., 1990). Human embryonic kidney 293 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12. At 24 h before transfection, cells were plated to a density of 5×10^6 per flask. Cells were transfected using the calcium phosphate precipitation method and harvested after 48 h (Gorman et al., 1990).

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Abbreviations used: NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

Control transfections were carried out with pSV- β -galactosidase and showed a transfection efficiency in the range 15–20% (Lim and Chae, 1989). Cells were collected by centrifugation (800 *g* for 5 min at 4°C), followed by homogenisation in ice-cold 50 mM Tris-acetate (pH 7.4) containing 5 mM EDTA and 5 mM EGTA (buffer 1) using a Dounce (glass/glass) homogeniser (20 strokes). The homogenate was processed by two cycles of centrifugation (120,000 *g* for 30 min at 4°C) and homogenisation (20 strokes) with final resuspension in buffer 1 and storage at –20°C.

Brain membrane preparation

Membranes from adult Wistar rat brains minus cerebellum were prepared as described by Hunter et al. (1990). In addition, the resulting pellets were washed a further three times by resuspension and centrifugation in buffer 1 (as above) at 4°C for 30 min at 58,000 *g*.

Radioligand binding assays

Radioligand binding assays were carried out by filtration using 1% (vol/vol) polyethyleneimine-soaked filters (Duggan and Stephenson, 1988). Routinely, brain membranes or 293 cell homogenates (50 μ g of protein) were incubated with 9 nM [³H]MK-801 in the presence of 10 μ M *L*-glutamic acid in buffer 1 for 2 h at 22°C. Nonspecific binding was determined in the presence of 10 μ M MK-801.

Antibody production and characterisation by immunoblotting

The peptide NMDAR1 (929–938), amino acid sequence LQLCSRHRRES, was coupled to keyhole limpet haemocyanin by the glutaraldehyde method, and polyclonal antibodies were raised in rabbits to the resultant conjugate (Duggan and Stephenson, 1989). Anti-peptide antibody production was monitored by enzyme-linked immunosorbent assay with peptide NMDAR1 (929–938) as the antigen. Maximal antibody titres of >1:50,000 were obtained, where the antibody titre is defined as the serum concentration that gives half the maximal absorbance at $\lambda = 492$ nm. Antibodies were affinity-purified on the NMDAR1 (929–938) peptide-CH Sepharose 4B affinity column (Duggan and Stephenson, 1990) with a yield of 1 mg of purified antibody/ml of immune serum.

Immunoblotting was carried out as described by Duggan et al. (1992) using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% gels under reducing conditions and with a final antibody concentration of 12 μ g of protein/ml. The specificity of the reaction was demonstrated by peptide blockade where purified antibodies were preincubated for 16 h at 4°C before immunoblotting with either 100 μ g of NMDAR1 (929–938)/ml in phosphate-buffered saline or phosphate-buffered saline only.

Deglycosylation of native and recombinant NMDAR1

Brain membranes (75 μ g of protein) or 293 cell homogenates (30 μ g of protein) were incubated in the presence or absence of *N*-Glycanase (final concentration, 20 U/ml) for 4 h at 37°C in 50 μ M sodium phosphate (pH 6.0) containing 0.4% (wt/vol) SDS, 20 mM EDTA, and 0.1% (vol/vol) β -mercaptoethanol. The reaction was terminated by addition of SDS-PAGE sample buffer, and samples were analysed by immunoblotting.

Protein quantification

Protein concentrations were determined by the method of Lowry et al. (1951), following precipitation with 10% (wt/vol) trichloroacetic acid with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Human embryonic kidney cells transfected with pCISNMDAR1 showed transient expression of NMDAR1, as demonstrated by both immunoreactivity with anti-NMDAR1 (929–938) antibodies and specific [³H]MK-801 binding activity (Fig. 1 and Table 1).

Thus, [³H]MK-801 bound specifically and to saturation to a single high-affinity site with a K_D of 8.5 ± 2.5 nM ($n = 3$) and a B_{max} of 113 ± 37 fmol of [³H]MK-801 binding sites/mg of protein ($n = 10$) in homogenates of kidney 293 cells transfected with pCISNMDAR1. This corresponded to 14,000–19,000 [³H]MK-801 binding sites per transfected cell (Table 1). No specific binding was detectable in non-transfected cells. The dissociation constant is similar to that determined in whole rat brain membranes (Table 1).

In immunoblots with brain membranes as antigen, anti-NMDAR1 (929–938) antibodies recognised a single immunoreactive species with M_r 117,000 \pm 2,000 ($n = 5$). This band was blocked by prior incubation of the antibody with NMDAR1 (929–938) peptide. With 293 cell homogenates as antigen, two immunoreactive species with M_r 117,000 and M_r 97,000 were detected in immunoblots. Both of these were blocked by preincubation with peptide. In control experiments using either rat liver homogenates (75 μ g of protein; data not shown) or nontransfected cells (Fig. 1), no immunoreactivity was detected in western blots. Thus, we assign the M_r 117,000 immunoreactive species as the NMDAR1 receptor polypeptide. This is consistent with both the predicted molecular weight from the deduced amino acid sequence (M_r 105,000), allowing for a contribution by weight for *N*-glycosylation (Moriyoshi et al., 1991; and see below), and with the identification of a M_r 120,000

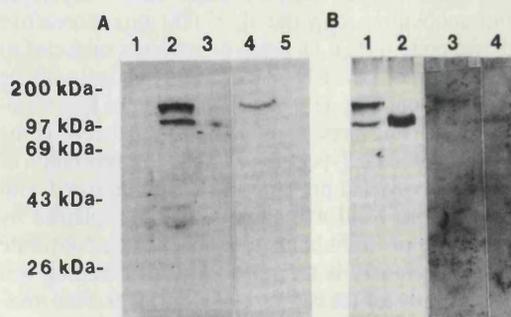


FIG. 1. Immunoblots of rat brain membranes and human embryonic 293 kidney cells transfected with NMDAR1, probed with anti-NMDAR1 (929–938) antibodies. Membranes prepared from adult rat brain minus cerebellum and transfected 293 cell homogenates were subjected to SDS-PAGE and immunoblotting using affinity-purified anti-NMDAR1 (929–938) antibodies (final concentration, 12 μ g/ml), all as described in Materials and Methods. **A:** Lane 1, nontransfected parent 293 cell homogenates (30 μ g of protein); lane 2, transfected 293 cell homogenates (30 μ g of protein); lane 3, as for lane 2 except that antibody was preincubated overnight at 4°C with NMDAR1 (929–938) (final concentration, 100 μ g/ml); lane 4, rat brain membranes (75 μ g of protein); and lane 5, as for lane 4 except that antibody was preincubated overnight at 4°C with NMDAR1 (929–938). **B:** Lane 1, control transfected 293 cell homogenates (30 μ g of protein); lane 2, transfected 293 cell homogenates (30 μ g of protein) following treatment with *N*-Glycanase as described in Materials and Methods; lane 3, rat brain membranes (75 μ g of protein); and lane 4, rat brain membranes (75 μ g of protein) following treatment with *N*-Glycanase. Positions and molecular weights of prestained protein standards are shown on the left.

TABLE 1. A comparison of the parameters for [³H]MK-801 binding to rat brain membranes and human embryonic kidney 293 cells transfected with pCISNMDAR1

Parameter	Brain membranes	Transfected cells
K_D (nM)	5.0 ± 0.8 (n = 2)	8.8 ± 2.5 (n = 3)
B_{max} (fmol of [³ H]MK-801 binding sites/mg of protein)	1,930 ± 30 (n = 2)	113 ± 37 (n = 10)

Radioligand binding assays were carried out as described in Materials and Methods. Scatchard analysis of binding isotherms was best fit by a single high-affinity site using the Enzfitter program (Elsevier-Biosoft, Cambridge, U.K.). Typical dpm values for the transfected cells were as follows: 2.0 nM [³H]MK-801, total dpm = 418 ± 7, nonspecific dpm = 289 ± 8; 27 nM [³H]MK-801, total dpm = 3,138 ± 10, nonspecific dpm = 2,697 ± 175. Data are mean ± SD values from triplicate determinations for the transfected cells and the mean ± range for brain membranes. For the B_{max} values for the cells, seven determinations are single point values from independent transfections corrected to B_{max} using the determined K_D value.

polypeptide in rat brain specifically photoaffinity-labelled with an azido derivative of MK-801 (Sonders et al., 1990).

The lower-molecular-weight species found in the transfected cells may be either a proteolytic fragment of the M_r 117,000 subunit or a differentially or unglycosylated form of the protein. Preparation of the cell homogenates in the presence of a series of protease inhibitors, including soybean trypsin inhibitor type II-S, bacitracin, benzamide hydrochloride (all at 10 µg/ml), and phenylmethylsulphonyl fluoride (0.1 mM), did not affect the pattern of immunoreactivity. However, following deglycosylation with *N*-Glycanase and immunoblotting, only the M_r 97,000 immunoreactive species was present (Fig. 1). Brain membranes subjected to the same treatment gave a band that was coincident with the M_r 97,000 subunit (Fig. 1). This suggests that the lower-molecular-weight band present in the transfected cells is the non-*N*-glycosylated polypeptide. The high concentration of the non-*N*-glycosylated protein in the cells compared with brain, where this band was absent, may be explained by overexpression of the subunit such that the carbohydrate processing machinery is saturated. A similar finding was reported recently for the expression of the rat skeletal muscle µI Na⁺ channel in the same 293 kidney cells (Ukomadu et al., 1992).

It is also noteworthy that in the immunoblots, the number of [³H]MK-801 binding sites from rat brain applied to each gel lane was 40-fold higher than for the transfected cells, yet the M_r 117,000 immunoreactive species was always enriched in the latter. The quaternary structure of NMDA receptors is not yet established. However, other classes of ionotropic glutamate receptor are heterooligomeric proteins (Wenthold et al., 1992). By analogy, it is not unreasonable to assume that such would be the case here. NMDAR1 must form functional homooligomers, albeit at low efficiency (Moriyoshi et al., 1991), which bind specifically [³H]MK-801 (see above), a phenomenon that is similarly found for γ-aminobutyric acid_A receptor α, β, γ, and δ subunits (Blair et al., 1988). Therefore, by analogy again, it is expected that related NMDA receptor subunits exist that stabilise oligomeric structure. Indeed, Meguro et al. (1992) recently described a novel glutamate receptor subunit, ε1,

that coassembles with the mouse homologue of rat NMDAR1 in *Xenopus* oocytes to give enhanced ion channel activity. Further studies using the above-described antibody together with antibodies raised against the ε1 protein will provide additional insights into native NMDA receptor structures.

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Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: a biochemical characterization

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The *N*-methyl-D-aspartate R1 (NMDAR1) and NMDAR2A subunits were expressed transiently either alone or in combination in human embryonic kidney (HEK) 293 cells. The biochemical and pharmacological properties of the cloned receptors were compared with those of adult rat brain NMDA receptors using both immunological methods with a newly developed anti-NMDAR2A-(1435–1445) antibody and [³H]MK801 radioligand binding activity. Anti-NMDAR2A-(1435–1445) antibodies recognized specifically four immunoreactive species with M_r s of 180000, 122000, 97000 and 54000 in rat brain, but only a single band of M_r 180000 in HEK 293 cells singly transfected with plasmid pCISNMDAR2A. N-deglycosylation of HEK cell membranes yielded a 165000- M_r immunoreactive species, which is in agreement with the size predicted from the cDNA sequence for the mature NMDAR2A subunit. Co-expression of NMDAR1

and NMDAR2A subunits in HEK 293 cells resulted in cell death. Thus conditions were established for the optimum expression of heteromeric receptors in viable cells, including a requirement for DL-2-amino-5-phosphonopentanoic acid (AP5) in the culture medium post-transfection. Cells transfected with pCISNMDAR1 and pCISNMDAR2A combined yielded a 10-fold increase in the number of [³H]MK801 binding sites compared with single subunit expression. MK801 had similar affinity for the expressed receptors as for those found in adult rat and mouse brain. These results demonstrate that the NMDAR1 and NMDAR2A receptor subunits co-assemble to form a heteromeric complex with properties similar to those of the native receptors of adult mammalian forebrain. Furthermore, the conditions reported for maximal transient expression provide a basis for further structure–activity studies.

INTRODUCTION

L-Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. It mediates its effects via interaction with the glutamate receptors, two main classes of which have been distinguished on the basis of their transduction mechanisms; namely the G-protein-coupled metabotropic and the fast-acting, ionotropic glutamate receptors (reviewed in Nakanishi, 1992). Within the ionotropic glutamate receptors, two pharmacological subclasses were defined: those activated by *N*-methyl-D-aspartate, i.e. the NMDA receptors, and those insensitive to NMDA, i.e. the non-NMDA receptors. The NMDA receptors are of particular interest because they are subject to multiple allosteric regulation by a strychnine-insensitive glycine binding site, polyamines and Mg²⁺ and Zn²⁺ cations. Additionally, they have been implicated both in the induction of long-term potentiation, an activity that may underlie learning and memory, and in neuronal degeneration (reviewed in Madison et al., 1991).

Until recently, there was little information available regarding the molecular properties of vertebrate NMDA receptors. In 1991, Moriyoshi et al. cloned by functional expression a cDNA encoding the NMDA receptor subunit NMDAR1. Multiple alternative splice variants of this subunit, together with a second subunit type, NMDAR2 (alternative nomenclature NMDAR ϵ 1), with isoforms 2A–2D, were later identified by cDNA homology hybridization (e.g. Kutsuwada et al., 1992; Monyer et al., 1992; Meguro et al., 1992; Ishii et al., 1993). Cloned NMDA receptors have been characterized electrophysiologically following the expression of different subunit combinations in *Xenopus* oocytes

or in mammalian cells (e.g. Kutsuwada et al., 1992; Monyer et al., 1992; Stern et al., 1992; Ishii et al., 1993). These studies, together with localization of the different mRNAs by *in situ* hybridization (e.g. Kutsuwada et al., 1992; Monyer et al., 1992), suggested that the native receptor is heteromeric and that it is probably composed of an NMDAR1 subunit in combination with either one of the NMDAR2 subunits in as yet unknown proportions.

In order to study the molecular properties of the NMDA receptors, it is important also to characterize both cloned and native receptors biochemically. The *Xenopus* oocyte system is not amenable for such studies, and therefore we have utilized expression in mammalian cells. Indeed, we have previously reported the expression and immunological characterization of NMDAR1 homo-oligomers, albeit expressed at low efficiency, in human embryonic (HEK) 293 cells (Chazot et al., 1992). In the present paper we now report the first biochemical characterization of NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells and, importantly, also, describe optimum conditions for their expression with minimal cell death.

MATERIALS AND METHODS

Materials

[³H](+)-5-Methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine (MK801; 28.8 Ci/mmol) was from Du Pont (U.K.) Ltd. (Stevenage, Herts., U.K.). The peptide NMDAR1-(929–938), amino acid sequence LQLCSRHRRES, was from Multiple Peptide Systems (San Diego, CA, U.S.A.). The peptides NMDAR2A-(1435–1445) (amino acid sequence YKKMPSIESDV),

Abbreviations used: AP5, DL-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DKA, 5,7-dichlorokynurenic acid; HEK, human embryonic kidney; MAPS, multiple antigen peptide system; MK801 (+)-5-methyl-10,11-dihydrodibenzo[a,d]cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate.

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NMDAR2B-(1446–1456) and the multiple antigen peptides (MAPs) YKKMPSIESDVAA-MAP and MAP-YKKMPSIESDV were obtained from Peptide and Protein Research (University of Exeter, Devon, U.K.); biotinylated anti-rabbit immunoglobulin, streptavidin–peroxidase complex, rabbit immunoglobulin, horseradish peroxidase-linked whole antibody and the Enhanced Chemiluminescence (ECL) detection system were from Amersham International (Amersham, Bucks., U.K.). N-Glycosidase F was from Boehringer Mannheim (Lewes, E. Sussex, U.K.). MK801 maleate was from Research Biochemicals Inc. (Natick, MA, U.S.A.); DL-2-amino-5-phosphonopentanoic acid (AP5), 5,7-dichlorokynurenic acid (DKA) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Tocris Neuramin (Bristol, U.K.). HEK 293 cells were a gift from Dr. T. G. Smart (School of Pharmacy, London, U.K.) and the pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, CA, U.S.A.). All other materials were from commercial sources.

Plasmid construction and cell transfection

The cDNA encoding the full-length rat NMDAR1 was excised from the pN60 plasmid (Moriyoshi et al., 1991) and cloned directionally into the *SmaI/XbaI* sites of the pCIS mammalian expression vector (Gorman et al., 1990) as previously described (Chazot et al., 1992). The cDNA encoding full-length mouse NMDAR ϵ 1 (NMDAR2) was subcloned into the pCIS via a two-step procedure. A 4.8 kb *BamHI/EcoRI* fragment of the cDNA encoding NMDA ϵ 1 was excised from pBKSA ϵ 1 (Meguro et al., 1992) and ligated into the *BamHI/EcoRI* polycloning site of pBluescript II KS+ to yield pBKSANMDAR ϵ 1-(138–1464). A 2.6 kb *XbaI/HindIII* fragment of the cDNA encoding NMDA ϵ 1 was excised from pBKSA ϵ 1 and cloned directionally into pCIS to yield pCISNMDA ϵ 1-(1–895). A 2.6 kb *HindIII/HindIII* fragment was excised from pBKSANMDAR ϵ 1-(138–1464) and ligated in the *HindIII* site in the correct orientation of pCISNMDA ϵ 1-(1–895) to yield pCISNMDA ϵ 1, which now contained the cDNA encoding the full-length NMDA ϵ 1 subunit. Note that there is an alternative nomenclature for the NMDA ϵ 1 subunit, NMDAR2A (Monyer et al., 1992) and this will now be used throughout the rest of this paper.

Cell transfection was performed as previously described (Chazot et al., 1992). Briefly, HEK 293 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12). At 24 h prior to transfection, cells were plated to a density of 4×10^6 per flask. Cells were transfected using the calcium phosphate precipitation method and subsequently grown in glutamine-free DMEM/F12 in the presence or absence of NMDA receptor ligands (Gorman et al., 1990). Cells were harvested up to 62 h post-transfection and processed as described (Chazot et al., 1992).

Brain membrane preparation

Forebrains were dissected from adult rats (Wistar strain) and adult mice (Balb/c strain), snap-frozen in liquid nitrogen and stored at -80°C until use. Membranes were prepared as described in Chazot et al. (1983).

Radioligand binding assays

[^3H]MK801 radioligand binding assays were performed using a polyethyleneimine filtration assay with $10\ \mu\text{M}$ MK801 for the determination of non-specific binding (Chazot et al., 1992). Numbers of binding sites stated in the text and Figure legends

are all B_{max} values. Where single point [^3H]MK801 radioligand concentrations were used, correction to B_{max} was made using the determined dissociation constants as in Table 3 or in Chazot et al. (1992).

Antibody production and characterization by immunoblotting

Anti-NMDAR1-(929–938) antibodies were generated and characterized as previously reported (Chazot et al., 1992). Polyclonal antibodies were raised in rabbits to the MAPS peptides, NMDAR2A-(1435–1445)-Ala-Ala-MAP and MAP-NMDAR2A-(1435–1445), i.e. peptides were linked to the branched lysine by both the N- and the C-terminus. Anti-peptide antibody production was monitored by an enzyme-linked immunoadsorbent assay with peptide NMDAR2A-(1435–1445) as the antigen (Stephenson and Duggan, 1991). Maximal antibody titres of 1:2500 were obtained, where the antibody titre is defined as the serum concentration that gives half the maximal absorbance at $\lambda = 492\ \text{nm}$. Antibodies were affinity-purified on the MAP-YKKMPSIESDV peptide–CH Sepharose 4B affinity column with a yield of $100\ \mu\text{g}$ of purified antibody/ml of immune serum (Duggan and Stephenson, 1990). The best results were obtained with the antigen NMDAR2A-(1435–1445)-AA-MAP, and this was thus used for all subsequent receptor characterizations.

Immunoblotting was carried out essentially as described by Duggan et al. (1991) using SDS/PAGE in 7% polyacrylamide mini-slab gels under reducing conditions and with a final antibody concentration of $10\ \mu\text{g}$ of protein/ml. Additionally, in some immunoblots, anti-(rabbit Ig) horseradish peroxidase-linked whole antibody was used in preference to the streptavidin peroxidase/biotinylated antibody complex. The specificity of the immunoreaction was demonstrated by peptide blockade where purified antibodies were preincubated for 16 h at 4°C before immunoblotting with $100\ \mu\text{g}$ of NMDAR1-(929–938), NMDAR2A-(1435–1445) or NMDAR2B-(1446–1456)/ml in phosphate-buffered saline, or with phosphate-buffered saline alone.

N-deglycosylation of recombinant NMDAR2A

Homogenates of HEK 293 cells transfected with NMDAR2A alone ($60\ \mu\text{g}$ of protein) were incubated in the presence or absence of N-Glycanase (final concentration 20 units/ml) for 4 h at 37°C in $50\ \mu\text{M}$ sodium phosphate, pH 6.0, containing 0.4% (w/v) SDS, 20 mM EDTA and 0.1% (v/v) β -mercaptoethanol. The reaction was terminated by addition of SDS/PAGE sample buffer, and samples were analysed by immunoblotting as described above. Control samples were treated exactly as above, except that the enzyme was omitted.

Protein determination

Protein concentrations were determined using the method of Lowry et al. (1951) with BSA as the standard protein.

Cell death

Cell death was determined by the uptake of Trypan Blue. Briefly, cells were harvested 20 h post-transfection, and centrifuged for 10 min at $1500\ g$ at 4°C . The pellet was resuspended in Hanks' balanced salt solution (10 ml). The number of cells which accumulated Trypan Blue was determined as a percentage of the total cell number using a haemocytometer according to the manufacturer's recommendations (Sigma Chemical Co., Poole, Dorset, U.K.).

RESULTS

Anti-NMDAR2A antibodies and their use in the detection of the NMDAR2A subunit in transfected cells and in rat brain

In a previous study, we described the generation of an anti-NMDAR1 antibody using as immunogen, the synthetic peptide NMDAR1-(929–938) coupled to keyhole limpet haemocyanin as a carrier protein. This antibody was used to immunologically identify the NMDAR1 glutamate receptor subunit expressed in HEK 293 cells and in adult rat brain. Anti-NMDAR1-(929–938) antibodies recognized specifically a band of M_r 117000 in immunoblots from cells transfected with pCISNMDAR1 which was coincident with that from adult rat brain membranes. Using the higher resolution SDS/7%-PAGE, the 117000- M_r seen in rat brain membranes was resolved into two species which differed by M_r 5000 (Figure 1b, lane 8). Additionally, in the transfected cells, a 97000- M_r immunoreactive species was also identified which was non-N-glycosylated NMDAR1 subunit (Chazot et al., 1992). We now report the production of anti-NMDAR2A antibodies adopting the multiple antigen peptide system (MAPS) approach (Tam, 1992). This method negates the use of a carrier protein for the production of antibodies against small synthetic peptides. Multiple peptides are instead covalently attached to a non-immunogenic branched lysine backbone and then used directly as the antigen (Tam, 1992).

In immunoblots with adult rat forebrain membranes, affinity-purified anti-NMDAR2A-(1435–1445) antibodies recognized four major species with M_r values of 180000 ± 4000 , 122000 ± 2000 , 97000 ± 2500 and 54000 ± 2000 ($n = 5$) (Figure 1a, lane 1; Figure 1b, lane 4). These bands were all blocked by prior incubation of the antibody with either the corresponding

MAPS or the free NMDAR2A-(1435–1445) peptide (Figure 1b, lane 6). With the HEK 293 cells transfected with pCISNMDAR2A alone, a single specific immunoreactive species of M_r 180000 ± 3000 ($n = 5$) was detected in immunoblots (Figure 1a, lanes 2 and 4; Figures 1b, lane 3). In control experiments using either rat liver homogenates or untransfected cells, no reactivity was detected in immunoblots (Figure 1b, lanes 1, 2). Following treatment with N-Glycanase of membranes prepared from HEK cells transfected with NMDAR2A, the M_r of the immunoreactive species was decreased to 165000 ± 3000 , compared with control samples which retained the M_r 180000 immunoreactive species (Figure 1a, lanes 4 and 5). The size of the non-N-glycosylated subunit is consistent with that deduced from the NMDAR2A cDNA (Meguro et al., 1992; Monyer et al., 1992). N-deglycosylation of rat brain membranes followed by immunoblotting resulted in the loss of immunoreactive bands in both tests and control samples, despite the inclusion of protease inhibitors.

Importance of NMDA receptor blockade for successful expression of heteromeric NMDAR1/NMDAR2A receptors in mammalian cells

In initial studies it was observed that, after 16 h, HEK cells co-transfected with pCISNMDAR1 and pCISNMDAR2A and cultured in DMEM/F12 media became detached from the culture dishes and lost viability compared with HEK cells transfected with either of the constructs alone. No immunoreactive bands were found in immunoblots using membranes prepared from the co-transfected HEK cells, in contrast to single subunit expression where M_r 117000 (NMDAR1) and M_r 180000 (NMDAR2A) immunoreactive species were observed (Figure 2). Furthermore, [3 H]MK801 binding activity was not detected in the co-transfected cells. Previous experience in transfection studies has revealed that successful transfections using HEK 293 cells were obtained with DMEM/F12 cell culture medium (results not shown). This medium is glutamine-free but it does contain

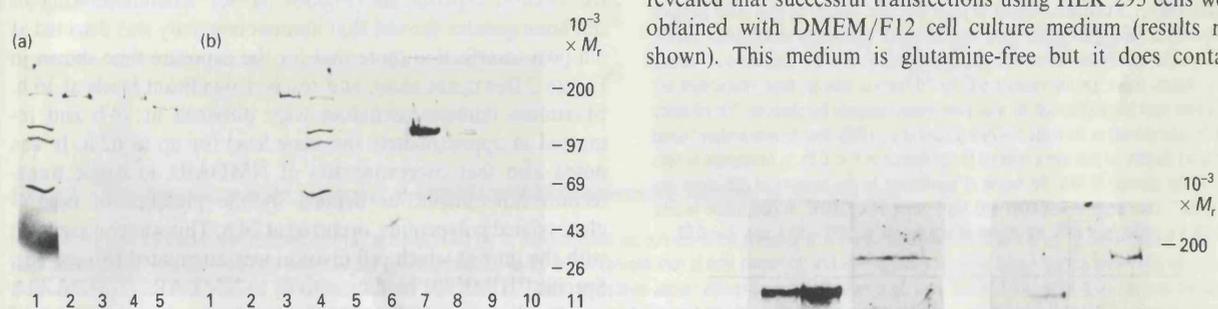


Figure 1 Immunoblots of rat brain membranes and HEK 293 cells transfected with pCISNMDAR1 and pCISNMDAR2A

Membranes were prepared from rat forebrain membranes and HEK 293 cells transfected with pCISNMDAR2A alone or with pCISNMDAR1 and pCISNMDAR2A in combination as described in the Materials and methods section. Immunoblotting was carried out by SDS/7%-PAGE under reducing conditions, transfer to nitrocellulose and using the ECL detection method. Affinity-purified antibodies were employed at final concentrations of $10 \mu\text{g/ml}$ anti-NMDAR1-(929–938) and $10 \mu\text{g/ml}$ anti-NMDAR2A-(1435–1445). (a) Blotting with anti-NMDAR2A-(1435–1445) antibodies only. Lanes: 1, rat forebrain membranes ($35 \mu\text{g}$ of protein); 2, HEK 293 cells transfected with pCISNMDAR2A alone ($60 \mu\text{g}$ of protein); 3, HEK 293 cells transfected with pCISNMDAR2A alone after preincubation of the antibody with NMDA-(1435–1445) ($100 \mu\text{g/ml}$) prior to immunoblotting ($60 \mu\text{g}$ of protein); 4, HEK 293 cells transfected with pCISNMDAR2A alone and incubated for 4 h at 37°C ($60 \mu\text{g}$ of protein); 5, as for 4 with the inclusion of N-glycosidase F in the incubation medium. (b) Lanes 1–6 show blots with anti-NMDAR2A-(1435–1445) ($10 \mu\text{g/ml}$) and lanes 7–10 show blots with anti-NMDAR1-(929–938) ($5 \mu\text{g/ml}$). Lane 1, liver membranes ($60 \mu\text{g}$ of protein); 2, non-transfected HEK 293 cells ($60 \mu\text{g}$ of protein); 3, 5, 7 and 9, HEK 293 cells transfected with pCISNMDAR1 and pCISNMDAR2A; 4, 6, 8 and 10, rat forebrain membranes; 11, prestained protein standards. For lanes 3–10, 60 fmol (B_{max} value) of [3 H]MK801 binding series per gel lane were applied. For lanes 5, 6, 9 and 10, the antibody was preincubated with the appropriate peptide ($100 \mu\text{g/ml}$) prior to immunoblotting.

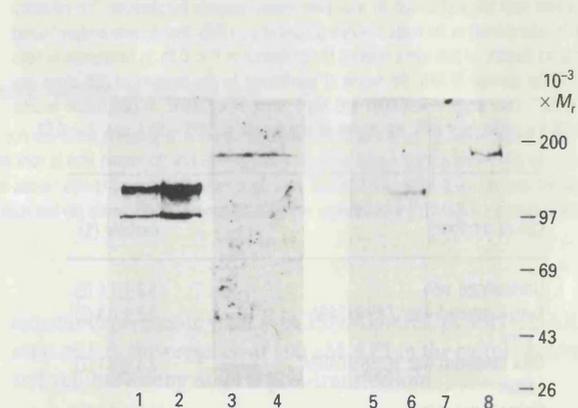


Figure 2 Demonstration by immunoblotting of the effect of AP5 in the culture medium following HEK 293 cell transfection with pCISNMDAR1 and pCISNMDAR2A

HEK 293 cells were transfected with either pCISNMDAR1 and pCISNMDAR2A alone or in combination, and subsequently grown in the presence or absence of AP5 ($200 \mu\text{M}$) for 48 h. Membranes were prepared from the transfected cells and immunoblotting carried out as described in the Materials and methods section. Lanes 1 and 2, cells transfected with pCISNMDAR1 alone and blotted with anti-NMDAR1-(929–938) antibodies; lanes 3 and 4, cells transfected with pCISNMDAR2A alone and blotted with anti-NMDAR2A-(1435–1445) antibodies; lanes 5–8, HEK 293 cells transfected with both plasmids and blotted with anti-NMDAR1-(929–938) antibodies (lanes 5 and 6) or with anti-NMDAR2A-(1435–1445) antibodies (lanes 7 and 8). Lanes 1, 3, 5 and 7, cells grown in the absence of AP5; lanes 2, 4, 6 and 8, cells grown in the presence of AP5. The positions of prestained protein standards are shown on the right.

sufficient concentrations of L-glutamate to open NMDA receptor channels with a predicted resultant influx of Ca^{2+} which may result in cell death. Thus, following co-transfection, HEK cells were grown in the presence of the NMDA receptor antagonist AP5 (200 μM); under these conditions the cells remained viable and both the NMDAR1 and NMDAR2A polypeptides were detected immunologically for up to 62 h. Similarly, [^3H]MK801 binding activity was detectable, with up to a 10-fold increase in binding with respect to HEK cells expressing the NMDAR1 subunit alone. Values ($\pm\text{S.D.}$; $n = 3$) obtained for fmol of [^3H]MK801 specific binding sites/mg of protein and corrected to B_{max} using K_D values as in Table 3 and Chazot et al. (1992) were: 90.4 ± 20.1 (pCISNMDAR1 alone); 45.2 ± 45.1 (pCISNMDAR1/pCISNMDAR2A in the absence of AP5); 1056.7 ± 20.0 (pCISNMDAR1/pCISNMDAR2A in the presence of AP5). No specific [^3H]MK801 binding was detected in HEK cells expressing only the NMDAR2A polypeptide.

Prevention of cell death by pharmacological manipulation

Since the NMDA receptor is modulated allosterically by different classes of compounds, a series of quantitative experiments was carried out in which the effect in the culture media post-cotransfection of the presence of different pharmacological agents, all known to antagonize NMDA receptor activation, was

investigated. Cell viability was monitored by Trypan Blue exclusion. The results are summarized in Table 1. Optimal results with respect to cell survival were obtained with a combination of AP5 and the antagonist of the strychnine-insensitive glycine site, DKA, where no significant difference between the percentage of viable cells in control and test samples was found. Mg^{2+} (10 mM) and DKA alone both partially protected against cell death. No effect was observed for the non-NMDA-receptor antagonist (CNQX).

The integrity of the NMDAR1 and NMDAR2A polypeptides and their assembly to form heteromeric receptors as determined by immunoblotting and [^3H]MK801 radioligand binding, was maintained under all of these protection conditions (results not shown). Indeed, with respect to these molecular properties, there was no difference observed between the presence of AP5 alone and that of AP5 plus DKA (results not shown). Routine transfection and culturing in the presence of 200 μM DKA is prohibitively expensive; therefore, since the molecular properties of the cloned receptor were unchanged under the two conditions, further optimization for the co-transfection studies was carried out in the presence of AP5 alone.

Determination of the optimum conditions for the expression of NMDAR1/NMDAR2A heteromeric receptors in mammalian cells cultured post-transfection in the presence of AP5

Because of difficulties experienced with toxicity as described above, the experimental conditions following HEK 293 cell transfection were investigated further in order to maximize the transient expression of NMDA receptors. Firstly, a time course for the expression of the receptors post-transfection was carried out (Figure 3). The time-dependent appearance of immunoreactivity was identical for both single subunit and co-transfection experiments (Figures 3a–3c). Immunoblotting of cell homogenates showed that immunoreactivity was detected at 3 h post-transfection (note that for the exposure time shown in Figure 3 this is not seen), and reached significant levels at 16 h. Maximum immunoreactivities were obtained at 24 h and remained at approximately the same level for up to 62 h. It was noted also that overexpression of NMDAR1 in single transfection experiments, as defined by the presence of non-N-glycosylated polypeptide, occurred at 24 h. This was concomitant with the time at which cell division was attenuated (Figure 3d). Specific [^3H]MK801 binding activity to NMDAR1/NMDAR2A expressed receptors followed a similar time course with a peak of binding sites by 24 h which remained stable for up to 62 h (Figure 3e).

The pCISNMDAR1/pCISNMDAR2A ratio used for transfection was also investigated. Earlier studies had shown that 10 μg of total plasmid DNA was optimum for the expression of receptors (results not shown). Therefore in all experiments the ratio was varied such that the total plasmid DNA used for cell transfection corresponded to this value (10 μg); the time of cell harvesting was 48 h. Both [^3H]MK801 binding assays and immunoblots were performed on the resultant transfected cells. The results for [^3H]MK801 binding are shown in Table 2. The immunoblots showed a profile which correlated directly with the amount of DNA of each respective subunit for transfection (results not shown). Maximum expression was achieved with pCISNMDAR1/pCISNMDAR2A ratios of 1:3 and 1:5. It was noted that, under these conditions, no overexpression of the NMDAR1 subunit, as defined by the detection of the non N-glycosylated polypeptide in immunoblots, occurred (Figure 3c).

In summary, the optimum conditions in the presence of AP5 post-transfection which were adopted as standard for all sub-

Table 1 The effect of NMDA receptor ligands on cell survival following co-transfection with pCISNMDAR1 and pCISNMDAR2A

HEK 293 cells were transfected by the calcium phosphate method with pCISNMDAR1 alone (10 μg), pCISNMDAR2A alone (10 μg) or co-transfected with both pCISNMDAR1 and pCISNMDAR2A (1:3 ratio with a total of 10 μg of DNA) and cultured for 20 h in the presence of different pharmacological agents. Cells were harvested, collected by centrifugation and the percentage cell viability determined by Trypan Blue exclusion as in the Materials and methods section. Values shown are the means \pm S.D. for 10 fields of view for three independent cell counts from each transfection and for n (in parentheses) separate transfections. The efficiency of cell transfection was in the range 15–20% (Chazot et al., 1992). Results were analysed using a two-tailed Student's t -test with a criterion of significance of $P < 0.05$. In comparison to cells grown in the absence of AP5, the degree of significance for the presence of AP5 alone was $P < 0.0001$; DKA alone, $P < 0.0001$ and Mg^{2+} alone, $P < 0.0002$. In comparison to cells grown in the presence of AP5, the degree of significance for AP5 + DKA was $P < 0.02$.

Culture conditions	Cell mortality (%)
Untransfected cells	3.3 \pm 1.8 (2)
Cells transfected with pCISNMDAR1 alone	3.2 \pm 0.9 (3)
Cells transfected with pCISNMDAR2A alone	2.4 \pm 0.1 (2)
Co-transfected cells	21.7 \pm 3.0 (11)
Co-transfected cells + 2 \times AP5 (200 μM each)	8.3 \pm 2.6 (5)
Co-transfected cells + 2 \times DKA (200 μM each)	12.8 \pm 3.7 (4)
Co-transfected cells + 2 \times Mg^{2+} (10 mM each)	13.6 \pm 3.7 (3)
Co-transfected cells + 2 \times [AP5 (200 μM) + DKA (200 μM)]	2.8 \pm 1.6 (3)
Co-transfected cells + 2 \times [AP5 (200 μM) + Mg^{2+} (10 mM)]	5.7 \pm 1.9 (4)
Co-transfected cells + 2 \times [DKA (200 μM) + Mg^{2+} (10 mM)]	5.2 \pm 1.8 (3)
Co-transfected cells + 2 \times CNQX (100 μM each)	21.8 \pm 4.6 (3)

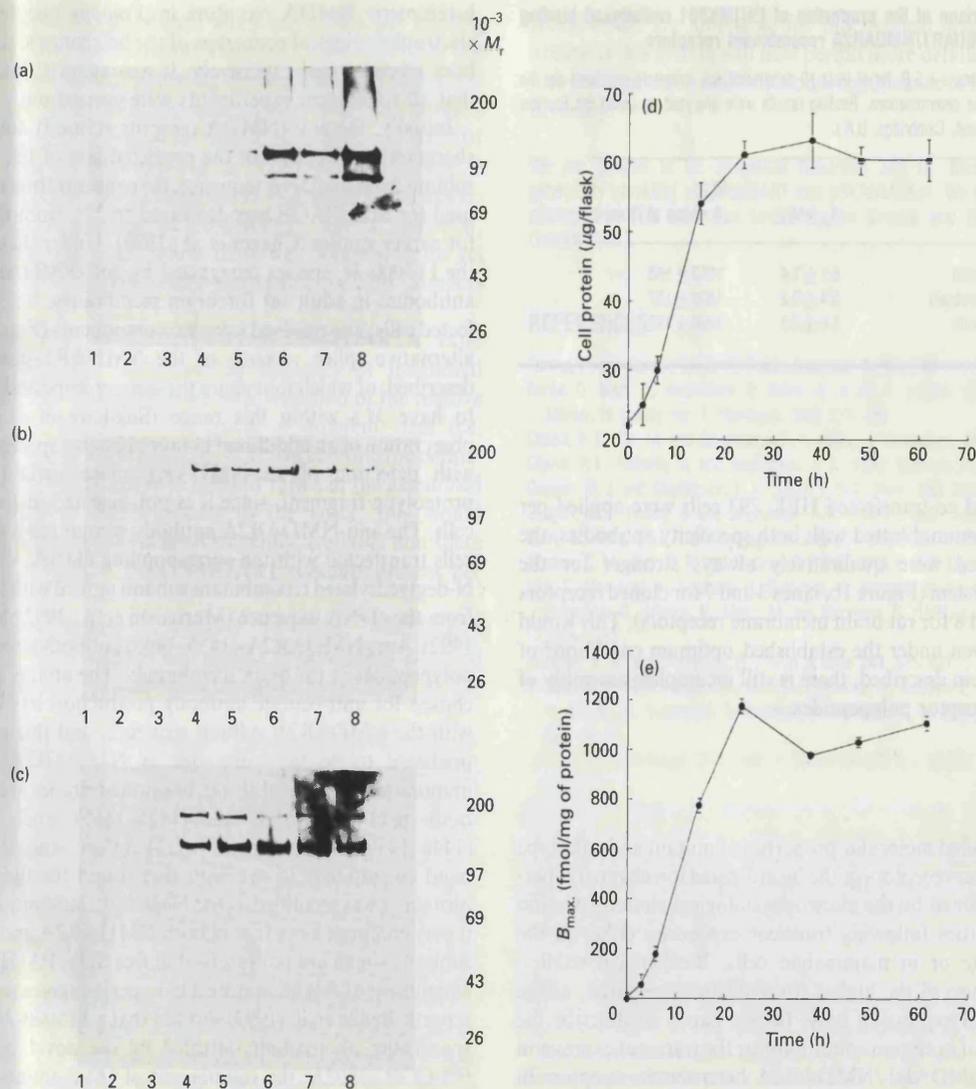


Figure 3 Time-dependence of the expression of NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells

HEK 293 cells were transfected with pCISNMDAR1 (a), pCISNMDAR2A (b) or both combined (c), cultured in the presence of AP5 and harvested at 0, 3, 6, 15, 24, 38, 48 and 62 h (lanes 1–8 respectively). At each time point, the cells were analysed by immunoblotting (a–c), for the concentration of total protein (d) and by [³H]MK801 specific ligand binding activity (e), all as in the Materials and methods section. (a)–(c) are immunoblots with transfection conditions as detailed above; affinity-purified antibodies used were anti-NMDAR1-(929–938) (a), anti-NMDAR2A-(1435–1445) (b) and both antibodies (c). (d) shows the time-dependence for cell protein concentration and (e) shows the time-dependence for the expression of [³H]MK801 radioligand binding activity, again with co-transfection conditions detailed above.

Table 2 Effect of varying the pCISNMDAR1/pCISNMDAR2A ratio used for co-transfection on the expression of heteromeric receptors in HEK 293 cells

HEK 293 cells were co-transfected with pCISNMDAR1 and pCISNMDAR2A in different ratios such that the total DNA used for transfection was always 10 μg. Cells were cultured post-transfection in the presence of AP5, harvested at 48 h and assayed for [³H]MK801 specific ligand binding activity, all as described in the Materials and methods section. Values are the means ± S.D. for three separate experiments.

Sample	Increase in [³ H]MK801 binding activity (fold)
NMDAR1 alone	1
NMDAR1/NMDAR2A, 1:1	2.7 ± 1.8
NMDAR1/NMDAR2A, 1:2	5.0 ± 1.1
NMDAR1/NMDAR2A, 1:3	9.0 ± 0.9
NMDAR1/NMDAR2A, 1:5	8.2 ± 0.3

sequent experiments were a pCISNMDAR1/pCISNMDAR2A ratio of 1:3, the presence of 200 μM AP5 in the culture medium, and cell harvesting at 24 h post-transfection.

The radioligand binding properties of cloned NMDAR1/NMDAR2A hetero-oligomers: comparison with native receptors

[³H]MK801 bound to NMDAR1/NMDAR2A heteromeric receptors at a single high-affinity site, with $K_D = 6.1 \pm 1.4$ nM and $B_{max} = 1053 \pm 185$ fmol/mg of protein ($n = 10$). The affinity of this site is comparable with that determined for the NMDA receptor in adult rat and mouse forebrain and for the singly expressed NMDAR1 subunit (Table 3; Chazot et al., 1992). The number of binding sites present in the co-transfection experiments showed an approximate 10-fold increase with respect to the NMDAR1 expressed alone. It was noted, however, that when equal numbers of [³H]MK801 binding sites for rat forebrain

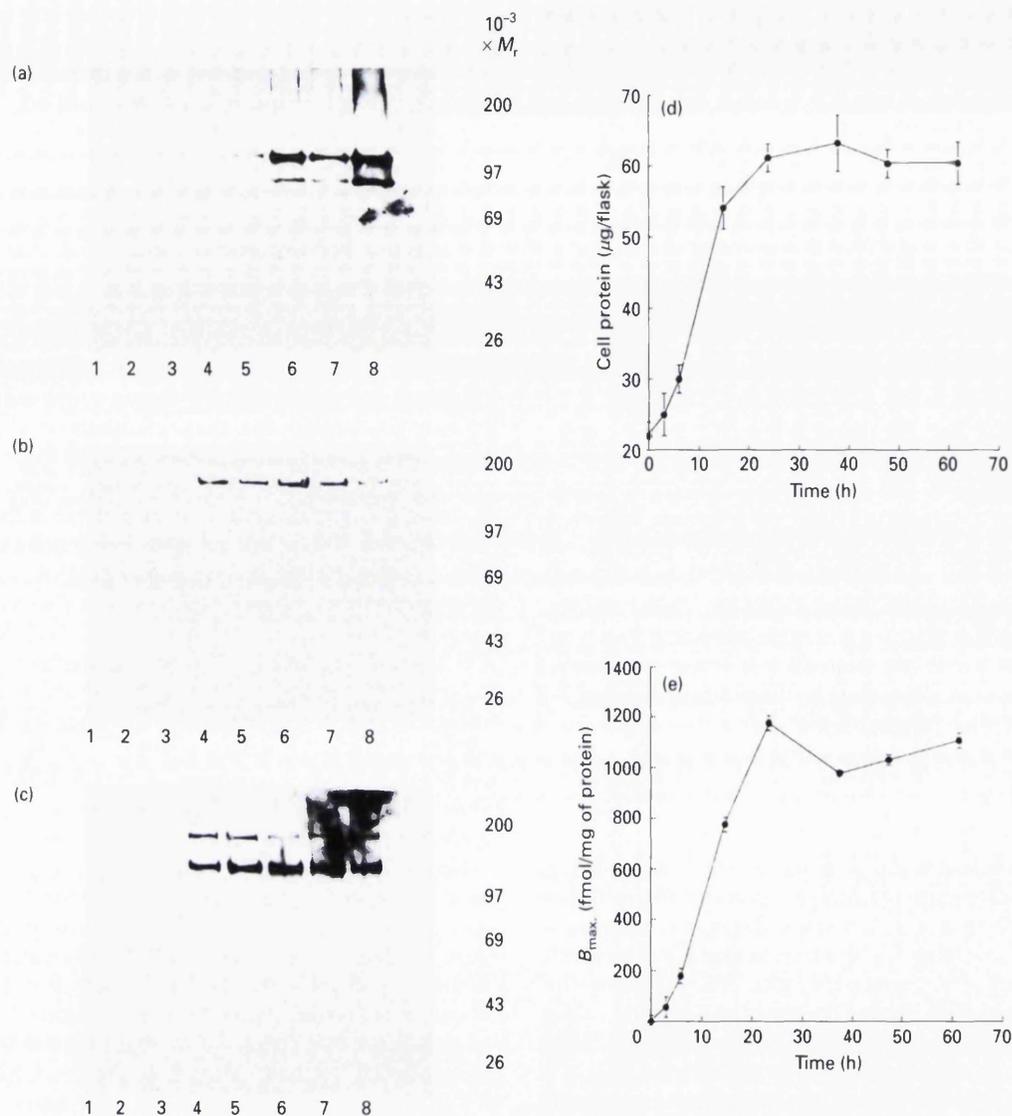


Figure 3 Time-dependence of the expression of NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells

HEK 293 cells were transfected with pCISNMDAR1 (a), pCISNMDAR2A (b) or both combined (c), cultured in the presence of AP5 and harvested at 0, 3, 6, 15, 24, 38, 48 and 62 h (lanes 1–8 respectively). At each time point, the cells were analysed by immunoblotting (a–c), for the concentration of total protein (d) and by [³H]MK801 specific ligand binding activity (e), all as in the Materials and methods section. (a)–(c) are immunoblots with transfection conditions as detailed above; affinity-purified antibodies used were anti-NMDAR1-(929–938) (a), anti-NMDAR2A-(1435–1445) (b) and both antibodies (c). (d) shows the time-dependence for cell protein concentration and (e) shows the time-dependence for the expression of [³H]MK801 radioligand binding activity, again with co-transfection conditions detailed above.

Table 2 Effect of varying the pCISNMDAR1/pCISNMDAR2A ratio used for co-transfection on the expression of heteromeric receptors in HEK 293 cells

HEK 293 cells were co-transfected with pCISNMDAR1 and pCISNMDAR2A in different ratios such that the total DNA used for transfection was always 10 µg. Cells were cultured post-transfection in the presence of AP5, harvested at 48 h and assayed for [³H]MK801 specific ligand binding activity, all as described in the Materials and methods section. Values are the means ± S.D. for three separate experiments.

Sample	Increase in [³ H]MK801 binding activity (fold)
NMDAR1 alone	1
NMDAR1/NMDAR2A, 1:1	2.7 ± 1.8
NMDAR1/NMDAR2A, 1:2	5.0 ± 1.1
NMDAR1/NMDAR2A, 1:3	9.0 ± 0.9
NMDAR1/NMDAR2A, 1:5	8.2 ± 0.3

sequent experiments were a pCISNMDAR1/pCISNMDAR2A ratio of 1:3, the presence of 200 µM AP5 in the culture medium, and cell harvesting at 24 h post-transfection.

The radioligand binding properties of cloned NMDAR1/NMDAR2A hetero-oligomers: comparison with native receptors

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Table 3 Comparison of the properties of [³H]MK801 radioligand binding to natural and NMDAR1/NMDAR2A recombinant receptors

B_{max} values are the means \pm S.D. for at least 10 determinations. Inhibitory constants are the means \pm S.D. for three determinations. Binding results were analysed by using the Enzfitter program (Elsevier-Biosoft, Cambridge, U.K.).

Source	K_D (nM)	B_{max} (fmol/mg of protein)
Recombinant	6.1 \pm 1.4	1053 \pm 185
Mouse forebrain	5.4 \pm 0.2	1809 \pm 157
Rat forebrain	5.0 \pm 0.8	1820 \pm 192

membranes and co-transfected HEK 293 cells were applied per gel lane and immunoblotted with both specificity antibodies, the signals obtained were qualitatively always stronger for the recombinant system (Figure 1b, lanes 3 and 7 for cloned receptors and lanes 4 and 8 for rat brain membrane receptors). This would suggest that even under the established optimum conditions of expression herein described, there is still incomplete assembly of the NMDA receptor polypeptides.

DISCUSSION

Currently, detailed molecular properties of mutant and wild-type neurotransmitter receptors of the ligand-gated ion channel superfamily are obtained by the electrophysiological characterization of their properties following transient expression either in the *Xenopus* oocyte or in mammalian cells. Biochemical studies, however, because of the higher concentrations required, utilize mammalian cell expression only. In this paper we describe the establishment of optimum conditions for the transient expression of wild-type NMDAR1/NMDAR2A heteromeric receptors in HEK 293 cells, thus providing a methodological base for further biochemical analyses of the molecular properties of this important neurotransmitter receptor protein. Such an investigation was required because it was found that the transient expression of NMDAR1/NMDAR2A receptors under standard transfection conditions resulted in rapid cell death and the loss of receptors, as measured both by [³H]MK801 radioligand binding activity and by immunoblotting with receptor-subtype-selective antibodies. The NMDA receptor channel is permeable to Ca²⁺; NMDA-receptor-mediated Ca²⁺ influx in the central nervous system is neurotoxic (reviewed in Rothman and Olney, 1987). At resting membrane potentials of -80 mV, the NMDA receptor channel is subject to blockade by Mg²⁺. Mg²⁺ ions are present at active concentrations (i.e. 0.7 mM) in the culture medium; however, the resting membrane potential of HEK 293 cells is in the range of -15 to -50 mV, potentials at which the voltage-dependent Mg²⁺ block is greatly reduced (Ascher and Nowak, 1987). Thus it was proposed that the mechanism of observed cell death in co-transfected cells compared with cells showing single subunit expression was due to high-level expression of functional receptors, activation by L-glutamate in the culture medium, sustained Ca²⁺ influx and resultant cell death. This was corroborated by the inclusion of the antagonist AP5 in the culture medium following co-transfection; this permitted cell survival, the biochemical characterization of the expressed receptor and a comparison with the properties of native receptors. Although not well documented, AP5 is also employed for expression of

heteromeric NMDA receptors in *Xenopus* oocytes, where the electrophysiological properties of the heteromeric receptors have been characterized extensively. It was against this background that all subsequent experiments were carried out.

Initially, the anti-(NMDA receptor subunit) antibodies were characterized. Because of the predicted size of the NMDAR2A subunit from its cDNA sequence, the concentration of acrylamide used for SDS/PAGE was decreased to 7% from the 10% used for earlier studies (Chazot et al., 1992). Under these conditions, the 117000- M_r species recognized by anti-NMDAR1-(929-938) antibodies in adult rat forebrain membranes, but not in transfected cells, was resolved into two components (Figure 1). Several alternative splice variants of the NMDAR1 gene have been described, of which four share the same epitope and are predicted to have M_r s within this range (Sugihara et al., 1992). The observation of an additional immunoreactive species is consistent with detection of an NMDAR1 splice variant and not a proteolytic fragment, since it is not detected in the transfected cells. The anti-NMDAR2A antibody recognized a single band in cells transfected with the corresponding cDNA. The size of the N-deglycosylated recombinant subunit agreed with that predicted from the cDNA sequence (Moriyoshi et al., 1992; Monyer et al., 1992). Anti-NMDAR2A-(1435-1445) antibodies recognized four polypeptides in rat brain membranes. The amino acid sequence chosen for anti-peptide antibody production has 73% identity with the NMDAR2B subunit sequence, and this polypeptide is predicted to be the same size as NMDAR2A. Indeed, the immunoreactive signal in rat brain membranes was blocked by both peptides NMDAR2A-(1435-1445) and NMDAR2B-(1446-1456) (results not shown). Therefore, since the 180000- M_r band corresponds in size with that found for the recombinant protein, it was identified as the NMDAR2 subunit. Alternatively, it may comprise a mixture of both NMDAR2A and NMDAR2B subunits which are not resolved in this SDS/PAGE system. The assignment of this immunoreactive species agrees with two recent reports. Benke et al. (1993) showed that a 175000- M_r protein was specifically photoaffinity-labelled by the novel NMDA ligand [¹²⁵I]-CGP 55802A, the sodium salt of (*E*)-2-amino-10-[(4-azido-2-hydroxy-3-iodobenzoyl)amino]-4-phosphonomethyldec-3-enoic acid. Tingley et al. (1993) reported that a 180000- M_r phosphorylated protein was co-immunoprecipitated with an anti-NMDAR1 antibody from primary cultures of rat cortical neurones. Both groups suggested that these may be the NMDAR2A and/or NMDAR2B polypeptides. The lower- M_r species that were immunoreactive with anti-NMDAR2A-(1435-1445) antibodies may be brain-specific proteins which share the same antigenic determinant as the NMDAR2 subunits. Alternatively, they may be splice forms of the NMDAR2A/2B genes that have not yet been discovered by cloning methodology.

The standard conditions recommended for mammalian cell transfection and transient expression of proteins (e.g. Sambrook et al., 1989) were not appropriate for NMDA heteromeric receptors because of toxicity problems (see the Results section). Therefore we established optimum conditions both for the expression of the respective polypeptides (determined immunologically) and for their co-assembly into functional receptors; the latter being assessed by [³H]MK801 binding activity. That the subunits do co-assemble to form heteromeric receptors was confirmed by the 10-fold increase in [³H]MK801 binding activity upon co-expression compared with single subunit expression. This is in agreement with both electrophysiological studies, where co-expression yielded enhanced L-glutamate-gated ion conductance (e.g. Kutsuwada et al., 1992; Monyer et al., 1992) and antibody studies for the non-NMDA receptors which demonstrated the existence of heteromeric receptors (Wenthold

et al., 1992). Low but significant levels of [³H]MK801 binding were detected for single-subunit NMDAR1 expression but not NMDAR2A expression, suggesting that the MK801 binding site is localized to the NMDAR1 subunit. In support of this, Sonders et al. (1990) reported that an azido derivative of MK801 specifically photoaffinity-labelled a 120000-*M_r* polypeptide (NMDAR1) in rat brain membranes. In that study there was no evidence for incorporation of radioactivity into a higher-*M_r* species (180000), although radioactivity was observed at the gel origin which may have represented aggregated receptor (Sonders et al., 1990).

Interestingly, the pCISNMDAR1/pCISNMDAR2A ratio used for transfection influenced the efficiency of functional receptor assembly. This may either be a reflection of the relative efficiency of the respective constructs of different sizes (i.e. pCISNMDAR1/pCISNMDAR2A, 1:1.5) in entering the permeabilized cells, or it may relate to subunit ratios in the assembled heteromeric protein. With respect to the latter, this would simply mean with the information currently available that there were more NMDAR2A than NMDAR1 subunits present per oligomer.

The pharmacological specificity of the NMDAR1/NMDAR2A cloned receptor was similar to that found in native forebrain compared to cerebellar receptors (e.g. Ebert et al., 1991).

The finding that levels of receptor expression in HEK 293 cells that were similar to those found in native brain membranes leads to cell death means that this parameter can be used as an independent index of efficient receptor assembly. Notably, blockade of the strychnine-insensitive glycine-binding site alone permitted a degree of cell survival in the co-transfected cells. This is further evidence to support a role of glycine as a co-agonist at the NMDA receptor (Kleckner and Dingledine, 1988). A further interesting point is that NMDA receptors are known to undergo desensitization and down-regulation by L-glutamate (summarized in Ascher and Nowak, 1987). Neuronal nicotinic receptors also desensitize, but they are up-regulated in response to chronic exposure to the agonist, nicotine (Wonnacott, 1990). In the experiments described here, clearly the continued presence of L-glutamate in the culture medium leads to no such adaptive response in either direction, which suggests a requirement for other factors and regulation at a gene level which is not permissive in the HEK 293 cells.

In summary, we have described in this paper the identification of the NMDAR2A receptor polypeptide in native and recombinant systems using a newly developed antibody. Additionally, we have reported the optimization of conditions for the expression of heteromeric NMDA receptors in mammalian cells at

levels which are amenable for biochemical analysis. The availability of this system will now permit more detailed investigations into the regulation and functional significance of NMDA receptor diversity.

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