RECEPTOR REGULATION OF CORTICAL PYRAMIDAL NEURONE ACTIVITY; IMPLICATIONS FOR THE TREATMENT OF ALZHEIMER'S DISEASE.

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science of the University of London.

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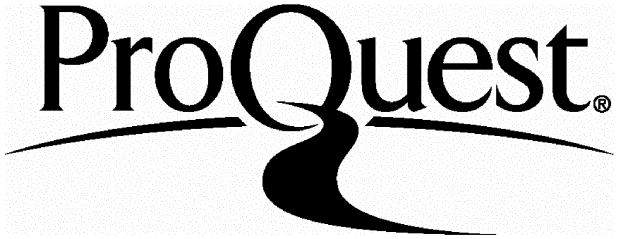

This thesis is dedicated to my parents, my wife and family, for the unending support and happiness they have given me throughout my life.

Abstract.

Pyramidal cells, the major neuron type in the cerebral cortex, are considered to play a pivotal role in Alzheimer's disease. Loss of pyramidal cells has been shown to not only to occur in the disease process and to correlate with a variety of measures of severity of dementia, but also to be the major locus of neurofibrillary tangle formation.

Pyramidal cells use excitatory amino acids as neurotransmitters. In order to investigate whether specific drugs, including a class in clinical use for Alzheimer's disease, can activate cortical neurones, an *in vivo* model was established using the intact corticostriatal pathway. This approach combines microdialysis with high performance liquid chromatography. In this model drugs are topically applied to the frontal cortex of the anaesthetised rat, and a microdialysis probe, positioned in the striatum, is used to measure increases in the extracellular concentration of excitatory amino acids glutamate and aspartate.

Using this model it was established that NMDA, topically applied to the frontal cortex increased concentrations in the striatum of both glutamate and aspartate. This effect was both tetrodotoxin sensitive and calcium dependent The selective 5-HT_{1A} antagonist WAY100135 modulated the NMDA-induced response. This effect was also tetrodotoxin sensitive and calcium dependent. The cholinesterase inhibitor physostigmine and the partial agonist at the M_1 receptor, PD 142505-0028, also increased extracellular concentrations in the striatum of glutamate but not aspartate. Additionally it was shown that the effect of PD 142505-0028 can be potentiated by WAY 100135. All these effects were tetrodotoxin sensitive and calcium dependent.

Preliminary data using the push-pull technique suggest that increasing extracellular concentrations of potassium, possibly mimicking depolarisation, increases amyloid precursor protein like-immunoreactivity in striatal push-pull perfusate.

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List of Abbreviations.

ACh	:	Acetylcholine
AChE	:	Acetylcholinesterase
ACPD	:	Aminocyclopentyl dicarboxylic acid
AD	:	Alzheimer's disease
AMPA	:	α-amino-3-hydroxy-5-methyl-isoxazole-4- propionic acid
ANOVA	:	Analysis of Variance
APLP1,2	:	Amyloid precursor like protein 1 and 2
APP	:	Amyloid precursor protein
APP_s	:	Secreted amyloid precursor protein
ApoE	:	Apolipoprotein E
Asn	:	Asparagine
ATP	:	Adenosine 5'-triphosphate
ßA4	:	ß amyloid peptide
Ca^{2+}	:	Calcium ion
$CaCl_2$	:	Calcium Choride
cAMP	:	Cyclic Adenosine Mono Phosphate
cDNA	:	Complementary deoxyribonucleic acid
ChAT	:	Choline acetyltransferase
CNS	:	Central Nervous System
CoA	:	Coenzyme A
COMT	:	Catechol-O-Methyl transferase
CSF	:	Cerebrospinal fluid

DAG	:	Diacylglycerol
DOPA	:	3,4-Dihydroxy-L-phenylalanine
DRN	:	Dorsal Raphe Nucleus
DTT	:	Dithiotreitol
EAA	:	Excitatory Amino Acid
e.g.	:	Exempli Gratia
EGTA	:	Ethyleneglycol-bis-(ß-amino-ethylether)-N,N,N',N'-tetraacetic acid
Fe^{2+}	:	Ferrous ions
FL	:	Forelimb area
$Fr_{1,2,3}$	:	Frontal area of the cortex (1,2, and 3)
GABA	:	γ Aminobutyric acid
GluR1-5	:	Family of glutamate receptor
GTP	:	Guanosine 5'-triphosphate
^{3}H	:	Tritiated hydrogen
5-HIAA	:	5-Hydroxyindoleacetic acid
HL	:	Hindlimb area
HPLC	:	High performance liquid chromatography
HRP	:	Horseradish peroxidase
5-HT	:	5-Hydroxytryptamine (serotonin)
$5\text{-}HT_{1A}$	:	The 1A type receptor of 5-HT
IP_3	:	Inositol-1,4,5-triphosphate
K^+	:	Potassium
Ka	:	Kainate
KCl	:	Potassium Chloride
kDa	:	Kilodalton

KPI	:	Protease inhibitor of the Kunitz type
Lys	:	Lysine
Leu	:	Leucine
LSD	:	Least square difference test
M_{1-5}	:	Muscarinic receptor type 1-5
m_{1-5}	:	Protein forming the muscarinic receptor (type 1-5)
mAChR	:	Muscarinic acetylcholine receptor
MAO	:	Monoamine oxidase
Meth	:	Methionine
Mg^{2+}	:	Magnesium
$MgCl_2$	:	Magnesium Chloride
MK801	:	(+)-5-Methyl-10,11-dihydro 5H-dibenzo [a,d] cyclohepten-5,10-imine (dizolcipine)
μm	:	Micrometer
MMSE	:	Mini mental state evaluation test
mPBS	:	Modified Phosphate Buffered Saline
mRNA	:	Messenger ribonucleic acid
Na	:	Sodium
NA	:	Noradrenaline
NaCl	:	Sodium Chloride
nbM	:	Nucleus basalis of Meynert
NFT	:	Neurofibrillary tangles
NMDA	:	*N*-methyl-D-aspartate
O_2	:	Oxygen
$Oc_{1,2}$	:	Occipital area of the cortex (1 and 2)
8-OH-DPAT	:	8-Hydroxy-2-(n-dipropylamino)-tetralin

$Par_{1,2}$	:	Parietal part of the cortex (1 and 2)
PC12	:	Pheochromocytoma cell line
PD 142505-0028	:	Azabicyclo [2.2.1] heptan-3-one, O-[3-(3-methoxyphenyl)-2-propynyl]oxime, (Z)-(=+/)-, ethanedioate (1:1) (salt)
PHF	:	Paired helical filaments
PKC	:	Protein kinase C
PLC	:	Phospholipase C
SD	:	Standard Deviation
SDS-PAGE	:	Sodium dodecyl sulfate-polyacryl-amide gel electrophoresis
TCP	:	1-[1-(2-thienyl) cyclohexyl] piperazine
TRIS	:	Tris (hydroxymethyl) methylamine
TTX	:	Tetrodotoxin
WAY 100135	:	N-tert-butyl 3-4 (2-methoxyphenyl) piperazin1-yl-2 -phenylpropanamidedihydrochloride

Chapter One

INTRODUCTION

1.1 Neurobiology of the Corticostriatal Pathway.

1.1.1 Evidence for the existence of the Corticostriatal Pathway

Cortical projections innervate the striatum in a topographic fashion resulting in a projection system with practically all parts of the cortex projecting to the immediately adjacent parts of the caudate-putamen. This system was first convincingly demonstrated in the rat (Webster, 1961) and has been repeatedly confirmed and extended in the rat and other species (Carman et al., 1963,1965; Cowan & Powell, 1966; Wise & Jones, 1977; Donoghue & Kitai, 1981; Gerfen, 1984;).

The cells of origin for the corticostriatal pathways are somewhat controversial. Jones and his colleagues (Jones et al., 1977; Wise & Jones, 1977) have argued that projections from the somatosensory cortex to the striatum come from small to medium sized pyramidal neurones in the superficial part of layer V and that these neurones project exclusively to the striatum, since their location does not coincide with any of the other known projection neurones to other cortical or subcortical sites. Donoghue & Kitai, (1981) on the other hand, have been able to demonstrate, with intracellular injections of horse radish peroxidase (HRP) in the rat, that some of the large pyramidal neurones sending main axons to other subcortical targets via the internal capsule, also send fine collaterals into the striatum. This confirmed earlier observations of Cajal (1909) using the Golgi technique, as well as suggestions of collateral projections on the basis of electrophysiological experiments (Endo et al., 1973; Miller, 1975; Jinnai & Matsuda, 1979). Using retrograde tracing techniques, Royce, (1982) has reported that corticostriatal projections in the cat arise from layers II, III, V and VI, and that at least the projections from layers V and VI to the striatum may be formed as collaterals of corticothalamic projections (Royce, 1983).

In the rat, Schwab et al., (1977) have reported retrograde labelling of neurones in cortical layers III to V following injection of tetanus toxin in the striatum. However, the dispersed nature of the internal capsule in the rat makes labelling of fibres of passage by retrograde tracers almost a certainty. Electrophysiological experiments in the rat are consistent with supragranular (Layers II and III) and infragranular (layers V and VI) origins of corticostriatal projections (Kitai et al., 1976).

1.1.2 Glutamate and Aspartate synthesis and metabolism.

The synthesis and metabolism of both glutamate and aspartate are compartmentalised in a very complex fashion. Compartmentalisation studies have indicated that a number of immediate precursors for glutamate synthesis exist: 1) from 2-oxoglutarate and aspartate by aspartate aminotransferase, 2) from glutamine by phosphate-activated glutaminase and 3) from 2-oxoglutarate by ornithine-aminotransferase (Cooper et al., 1991). The relative contribution of glutamine or glucose to transmitter glutamate synthesis is still an open question. Glutamate and aspartate are metabolically coupled through aspartate aminotransferase and this equilibrium between them depends on the availability of glucose and other precursors (Fonnum et al., 1986; Szerb & O'Regan, 1987; Burke & Nadler, 1989).

1.1.3 Evidence that glutamate and aspartate are neurotransmitters

Before being recognised as a neurotransmitter, a given substance has to meet certain criteria (Werman, R. 1966).

1) It is localised presynaptically in a neuron which uses it as a neurotransmitter.

2) It is released by physiological stimuli in a Ca^{2+}-dependent manner.

3) Its action mimicks that of the naturally occurring transmitter and can be blocked by specific antagonists.

4) Selective uptake mechanism exist which will remove the substance from the synaptic cleft.

1) Detailed electron microscopic studies using ultrathin sections for the study of glutamate (GLU) immunoreactivity over subcellular organelles within terminals have shown a localisation of GLU immunolabelling over synaptic vesicles and mitochondria (Ottersen, 1991). However this does not seem to be the case for aspartate (Zhang et al., 1990). Another approach is to use ultra-thin sections and colloidal gold particles as immunocytochemical markers, which allow the quantification of glutamate-like immunoreactivity in different cell compartments (Somogyi et al., 1986; Ottersen, 1987, 1989). Studies using these techniques show that nerve terminals of presumed glutamatergic neurons contain several fold higher particle densities than presumed GABA-ergic terminals, and that they are also enriched in immunoreactivity relative to the parent cell and the surrounding tissue average. Immunolabelling is concentrated over synaptic vesicles, and over mitochondria, the main site of glutamate synthesis, and relatively low over organelle-free axoplasm (Ottersen et al., 1990,b). This strategy has been used to demonstrate an enrichment of glutamate in mossy and parallel fibres of the cerebellum (Somogyi et al., 1986; Otterson 1987;1989), terminals of the perforant path, commissural/Schaffer collaterals, dentate associational/commissural fibres and mossy fibres of the hippocampus (Ottersen, 1989; Bramham et al., 1990; Ottersen et al., 1990,b), bipolar cell terminals (Ehinger et al., 1988), retinal ganglion cell terminals in the lateral geniculate body (Montero & Wenthold, 1989), primary afferent terminals in the spinal cord (Maxwell et al., 1990 a,b) and terminals of the spinocervical tract (Broman et al., 1990).

Whereas immunoelectron microscopic investigations at the nerve terminal level have provided ample evidence in support of a neurotransmitter role of glutamate in many

excitatoryfibre systems, a very different situation exists for aspartate. Immunogold analyses of perfusion fixed material from the spinal cord (Maxwell et al., 1990,b) and several brain regions have revealed very low levels of aspartate immunoreactivity in nerve terminals in which aspartate has been strongly suspected to have a transmitter role, such as the climbing fibre terminals of the cerebellum (Zhang et al., 1990).

2) Both ASP and GLU are released by chemical and electrical depolarising signals in various brain regions (see for review: Fonnum, 1984; Nicholls 1989).

An essential property of EAA terminals is their ability to release the transmitter in a Ca^{2+} dependent manner. Evidence has been accumulating that the Ca^{2+}- dependent pool of glutamate is indeed localised in synaptic vesicles (for reviews see: Nicholls, 1989; Maycox et al., 1990). For example, highly selective uptake systems for glutamate have been demonstrated in synaptic vesicle membranes (Naito & Ueda, 1985). Also biochemical experiments using synaptosomes have gained evidence for the existence of two intraterminal pools of releasable glutamate, one of which is Ca^{2+} dependent, possessing the typical osmotic resistance characteristic of synaptic vesicles and which equilibrates slowly (Nicholls, 1989), and one Ca^{2+}-independent, located in the cytoplasm, that equilibrates readily with exogenously applied glutamate. Synaptic vesicles have been shown to be able to maintain a high glutamate concentration provided a proton gradient is maintained across the vesicular membrane during isolation (Burger et al., 1989). Evidence which speaks against this assumption is for example that aspartate does not seem to be transported into synaptic vesicles (Naito & Ueda, 1985; Maycox et al., 1990). In addition recent studies have failed to demonstrate a significant Ca^{2+}-dependent release of D-aspartate (and endogenous aspartate), from a synaptosomal preparation, even when a Ca^{2+}-dependent glutamate release could be shown (Nicholls, 1989). Ca^{2+}-dependent release of exogenous and endogenous aspartate is obtained more consistently in brain slices (e.g. Nadler et al., 1976;1978; Toggenburger et al., 1983; Szerb, 1988) than in synaptosomes. However, cerebellar and hippocampal slices display a Ca^{2+}-

dependent release of endogenous aspartate, which is reduced after deprivation of climbing fibre or commissural fibre input, respectively (Nadler et al., 1976;1978; Toggenburger et al., 1983; Vollenweider et al., 1990). In contrast to this, work by McMahon et al. (1992) suggests that tetanus toxin, which is believed to interfere with fusion of the synaptic vesicle membrane with the presynaptic membrane, inhibits the release of both GLU and ASP in guinea-pig cerebrocortical synaptosomes, strongly suggestive of a neurotransmitter role for both EAA's. Also in hippocampal slices and CA1 minislices 50 mM potassium induced a Ca^{2+}-dependent release of both GLU and ASP (Fleck et al., 1993).

3) Both GLU and ASP act on post-synaptic receptors, commonly divided into ionotropic and metabotropic types (Mayer & Westbrook, 1984). Two subtypes of ionotropic EAA receptors, the AMPA/Kainate and the NMDA receptor are largely colocalised at EAAergic synapses (Jones & Baughman, 1991). While GLU is though to be a potent agonist at all EAA receptors, ASP appears to be selective for NMDA receptors (Olverman, 1988; Verdoorn & Dingledine 1988; Parneau & Mayer, 1990)

The N-methyl-D-aspartate receptor ionophore complex (NMDA receptor).

NMDA gates a channel with relatively slow kinetics, and high Ca^{2+} permeability. Another characteristic of the NMDA receptor is the voltage dependent block executed by the Mg^{2+}-ion. In addition a complex of regulatory sites have been identified, which may be affected by a number of agents (Cooper et al., 1991). The NMDA receptor appears to be involved in neurotoxicity and processes related to learning and memory. Some autoradiographic studies have suggested the existence of multiple forms of the NMDA receptor with distinct pharmacological properties. This may reflect a two-state model of high and low affinity agonist binding sites (Fagg & Massieu, 1991), or different combinations of the subunits NR1 and NR2A-NR2D (Moriyoshi et al., 1991;

Seeburg, 1993; Hollman & Heinemann, 1994).

Patch-clamp studies suggested that glycine, in submicromolar concentrations increased the channel opening frequency of the NMDA receptor (Johnson & Ascher, 1987). It has become clear that glycine is required for receptor activation, and is now known as a co-agonist (Lodge & Collingridge, 1991; Palfreyman & Baron, 1991).

A number of "dissociative anaesthetic" compounds bind to the open channel of the NMDA receptor, including phencyclidine and its derivative TCP, ketamine and the anticonvulsant dizolcipine (MK-801), (Foster, 1991). Binding of such non-competitive compounds is use dependent, and as such provide a measure of NMDA activation (Foster & Wong, 1987; Huettner & Bean, 1988).

Polyamines increase the binding of [^{3}H] MK-801 induced by glutamate and glycine (Ransom & Stec, 1988; Williams et al., 1991). Spermine and spermidine act as agonists, and putrescine as an antagonist (Williams et al., 1991). In addition to the regulatory sites discussed above, the NMDA receptor may also be blocked by low concentration of Zn^{2+}, a mechanism independent of the voltage dependent Mg^{2+} block (Westbrook & Mayer, 1987). Low concentrations of arachidonic acid have also been recently demonstrated to affect NMDA receptor activation, possibly via an intracellular response (Kwak et al., 1992).

The Kainate receptor

It is only recently that the kainate receptor is recognised as being a separate receptor from the AMPA receptor, as many studies were unable to link a given response of one agonist to one specific receptor (Patneau & Mayer, 1991; Cai & Erdo, 1992). However, cloning, expression and examination of the function of kainate receptors in xenopus oocytes (Egebjerg et al., 1991; Werner et al., 1991) has led investigators to assume that two different receptors were functioning in the brain. Kainate receptors are made out of the gene products GluR5, GluR6, GluR7 (low

affinity kainate receptor) (Hollman & Heinemann, 1994) and KA1 and KA2 (high affinity kainate receptor) (Seeburg, 1993). Kainate receptors display regional heterogeneity compared to AMPA receptors. The most striking difference is the lack of expression of the kainate receptor in CA1 of the hippocampus, and its very high level of expression in CA3. However, certain isoforms of both AMPA and kainate receptors may gate Mg^{2+} and Ca^{2+} in addition to monovalent cations when activated. Pharmacological differentiation of kainate and AMPA receptors is being facilitated by the development of more selective ligands (Kwak et al., 1992).

The AMPA receptor

AMPA receptors can be reconstituted *in vitro* by expressing one, or co-expressing any two of the four subunits termed GluR1-GluR4 (Seeburg, 1993; Hollman & Heinemann, 1994). These subunits occur in two major forms by alternative splicing. The "flip" and "flop" forms are differentially expressed in the developing as compared to the mature mammalian brain. Developing brain expresses flip forms only, whereas a co-expression of both forms appear in the mature brain. However up till now, pharmacological differentiation of the two is impossible. As described, it is now accepted that kainate and AMPA receptors are different entities. They may easily be distinguished electrophysiologically. Both kainate and AMPA receptors display different electrophysiological characteristics from that of NMDA receptors; the former receptors activate channels with fast kinetics (onset, offset and desensitisation measured in milliseconds), while NMDA receptors show much slower kinetics.

The metabotropic receptor

Apart from the ionotropic glutamate receptors, a so called metabotropic glutamate receptor family has been identified, which are linked to a G-protein mediated second messenger system

(Heinemann et al., 1991a; Hollman & Heinemann, 1994), There is virtually no sequence homology between mGluR and other G-protein linked receptors. A number of pharmacological agents show activity at the metabotropic receptors; the actions of quisqualate and trans-ACPD (both agonists) were central to the identification of the metabotropic receptor class.

When expressed in oocytes, both the mGluR1 and mGluR5 metabotropic receptors stimulate the PLC signal transduction mechanism, generating the second messengers IP_3 and DAG. Activation of these receptors produces large, long lasting oscillating currents by Ca^{2+} dependent mechanisms (Hollman & Heinemann, 1994). Membrane depolarisation alone is not sufficient to trigger the mGluR signal transduction pathway. Recently, the mGluR1 receptor has been demonstrated to couple with cAMP and arachidonic acid formation through G_s (Aramori & Nakanishi, 1992). The latter may be implicated in NMDA receptor function.

The mGluR2-mGluR4, mGluR6 and mGluR7 metabotropic glutamate receptors are negatively linked to adenylyl cyclase through G_i. In addition, the mGluR2 receptor slightly affects the PLC signal transduction pathway (Tanabe et al., 1992). Activation of these receptors produces an inhibitory response. Further investigation of the localisation and physiological properties of all metabotropic receptors awaits development of selective agonists and antagonists.

4) EAA's are taken up from the extracellular space into neurones and glial cells against large concentration gradients by Na-EAA cotransporters (Erecinska, 1987). There appears to be one high affinity uptake systems specific for acidic amino acids, which takes up L-glutamate and L-aspartate (see for review: Balcar & Li, 1992).

1.1.4 Evidence that the Corticostriatal Pathway uses Glutamate and Aspartate as neurotransmitters.

The following approaches have been used to investigate the neurotransmitters used by the

corticostriatal pathways. They generally support the concept that the corticostriatal pathway uses GLU and ASP as its neurotransmitters (as reviewed in Ottersen, 1991).

1) High-affinity uptake in fresh brain slices maintained *in vitro* can be visualised by autoradiography, both at the light and at the electron microscopic level (Storm-Mathisen & Iversen, 1979). In combination with selective lesions, this procedure has given precise information about the trajectories of EAA pathways (Taxt & Storm-Mathisen, 1984).

2) Instead of using slices that are fixed immediately after exposure to the radiolabelled substrate it is also possible to administer the substrate to the brain in an *in vivo* setting, and allow a survival time of several hours before fixation and preparation of the brain for autoradiography. This strategy permits time, not only for the uptake into the specific nerve terminal but also for retrograde transport of the tracer to the respective cell bodies (Streit, 1980). Such "transmitter selective retrograde tracing" has contributed greatly to the mapping of EAA pathways, particularly at the subcortical level. The use of the uptake-carrier to identify transmitters brings with it certain disadvantages. For example, it is not possible with this method to differentiate between glutamatergic and aspartergic terminals. Also glial cells are capable of glutamate uptake and may cloud the picture even more.

3) Uptake of radiolabeled glutamate and aspartate can be recorded biochemically in synaptosome preparations (Fonnum, 1984). A reduced uptake in synaptosomes prepared from the target area of a lesioned pathway is strong evidence for the involvement of glutamate or aspartate and has pointed to glutamate as a likely transmitter in a number of corticofugal connections. Particularly studies on ablation of the frontal cortex have shown a reduced EAA neurotransmitter function in the neostriatum (e.g. McGeer et al., 1977)

4) *In vivo* studies using push-pull in decorticated rats, observed a reduction in the potassium-evoked release of both GLU and ASP in the striatum (Girault et al., 1986)

1.1.5 Glutamate and excitotoxicity

The history of excitotoxicity began with the discovery by Hayashi ,(1952) that the amino acids glutamate and aspartate could produce convulsions when injected into the cortex. Five years later, Lucas and Newhouse (1957) reported the toxic effects of glutamate and aspartate when they administered these compounds subcutaneously to mice and observed subsequent damage to the inner layer of the retina. Another important finding was the demonstration of Curtis et al., (1959) that aspartate and glutamate have potent excitatory actions when applied iontophoretically to neurones in the spinal cord of cats. In that same year van Harreveld, (1959) noticed that topical application of glutamate to the cerebral cortex produced spreading depression. Curtis & Watkins, (1960) subsequently published the results of a series of experiments in which they were able to dissociate excitatory from inhibitory actions of different amino acids. Work by Fifkova & van Harreveld, (1970) indicated that the effects of intravenous glutamate administration resembled the consequence of spreading depression and asphyxia. Based on these observations they proposed that spreading depression may be caused by the release of glutamate from intracellular stores. In the next year Van Harreveld & Fifkova, (1971) published a paper describing light- and electron-microscopic changes in the central nervous system after electrophoretic injection of glutamate. This was the first time that glutamate was reported to cause the formation of dark cells with halos around them which consisted of grossly swollen tissue elements. Olney and his colleagues pursued this line of research further and in 1971 they detailed the cytotoxic effects of sulphur-containing and acidic amino acids in the central nervous system of the infant mouse. From these studies they concluded that there was a correlation between the neurotoxic effects of certain amino acids and their ability to excite neurones. Furthermore they postulated that increases in the concentrations of natural acidic amino acids could lead to neurodegenerative disease. The same group extended these observations with a paper in 1974 in which they reported the effects of kainic acid, a powerful

neurotoxic analogue of glutamate. An important concept in both these papers (Olney et al., 1971,1974) was the portrayal of the postsynaptic nature of the toxic changes. Olney summarised the neurotoxic effects of the different amino acids in a review four years later (Olney, 1978) and it was here that he coined the term excitotoxicity for the first time to include both the excitatory and toxic nature of the effect of these amino acids on neurones. The next major step forward in research on excitatory amino acids came in 1983 with a paper by Rothman, (1983). He proposed, on the basis of experiments with cultured hippocampal neurones, that synaptic activity mediated the death of neurones which had been starved of oxygen. Later (1984) he showed that it was the synaptic release of excitatory amino acids which caused the neuronal death. Furthermore, Benveniste et al., (1984) demonstrated by microdialysis that the concentrations of glutamate and aspartate increased during forebrain ischemia in animals, whilst Meldrum and coworkers (reviewed in Meldrum, 1990) showed that blockade of the NMDA receptors could protects against the development of ischaemic brain damage in the brains of animals. These findings provided the framework for the scientific case for the neurotoxic effects of excitatory amino acids. Glutamate and aspartate are released during ischemia and anoxia, their action causes excessive postsynaptic excitation and cell death, and antagonists of the NMDA subtype of excitatory amino acid receptor can prevent ischemic damage. This prompted Rothman & Olney , (1986)to suggest that glutamate plays a key role in the development of ischemic damage, and that drugs which block the effects of glutamate may provide a rational therapy for stroke (Rothman, 1986). Work by Henneberry has allowed an extension of the excitotoxicity concept to other, slowly developing neurodegenerative diseases, like Huntington's disease and AD. He showed that in conditions where intracellular energy levels are reduced, the magnesium block of the NMDA receptor is removed, rendering the neurone more vulnerable to excitatory amino acids (Henneberry, 1989).

1.2 Morphology of the cortex

1.2.1 Morphology of the mammalian neocortex.

Almost the entire cortex of sub-mammalian vertebrates is olfactory in nature, and contains only three layers of cells. As derivatives of this allocortex the paleocortex and the archicortex of the mammalian brain have evolved. The archicortex is incorporated in the limbic system and is present in the hippocampus and dentate gyrus. The neocortex, which consists of six layers, increases in size as higher mammals evolve, mainly due to the fact that progressively larger areas of association cortex develop with increasing interneuronal connection complexity (Barr, 1974).

The neocortex has cell bodies and fibres within it arranged in six layers. These laminae are as follows (Warwick & Williams, 1973)

Layer I) *The molecular layer* is situated immediately underneath the pia. It consists of horizontal cells of Cajal, and a dense network of fibres which run tangential to these neurones, and which are derived from pyramidal cells (apical dendrites), stellate cells (vertical axons) and other elements, including cortical afferent fibres, both projection and associational.

Layer II) *The external granule layer* contains the somata of stellate and small pyramidal cells. Passing through this lamina are vertically arranged dendrites and axons from subjacent layers, and a dense neuropil of local dendrites and axons.

Layer III) *The pyramidal layer* (supragranular) consists of pyramidal cells of medium size, stellate and fusiform cells, whose dendrites and axons extend far beyond the layer itself.

Layer IV) *The internal granule layer* consists mainly of stellate cells, with a number of small pyramidal cells. A prominent band of horizontally arranged fibres is also a feature of this layer, which contains a large number of vertically organised fibres.

Layer V) *The ganglionic layer* (infragranular) contains the largest pyramidal cells in a given

area. A small number of stellate cells may also be observed, and there is also a considerable amount of horizontally organised fibres.

Layer VI) *The multiform or fusiform layer* contains a variety of cell types, mostly small in size and thought to be modified pyramidal cells. This lamina is not always well demarcated from the adjacent cortical zone of fibres going to or coming from the cortex itself.

Functional columnar circuitry is one of the fundamental characteristics of the neocortex. This columnar principle essentially states that all neurones in a narrow column, perpendicular to the pia, and approximately 500 μm wide, are functionally coupled (Mountcastle, 1957; Hubel & Wiesel, 1965; Hubel & Wiesel, 1974).

From a histological point of view the structure of the neocortex is similar in all mammals. Pyramidal neurones are the most numerous in the neocortex, accounting for almost 70 % of the neuronal count (Winfield et al., 1980), with non-pyramidal accounting for the remainder. A basic uniformity in the mammalian neocortex has been demonstrated by a number of workers. For example, Sloper et al., (1979) found the absolute number of neurones and the ratio of pyramidal to stellate cells was similar in two different areas of primate brain although the thickness of the neocortex differed. Throughout the cortical laminae, there is also a large population of neuroglial cells (astrocytes, oligodendrocytes and microglia) and a close capillary network.

Pyramidal neurones of the neocortex fall into three main categories on the basis of their projections: corticofugal projection neurones project to subcortical targets, association neurones form homolateral connections with other neocortical neurones, and commissural neurones project to other neocortical neurones but in the opposite hemisphere. Projections of neocortical neurones within the cortex are referred to corticocortical (Zilles & Wree, 1985).

1.2.2. The areal structure of the rat neocortex.

In his studies on the areal and laminar structure of the human neocortex, Brodmann formulated three main postulates for the definition of a given cortical area: (I) The structure of a given area should be uniform; (ii) The area should be identifiable in related forms of mammalian brain; (iii) The main regions should have a constant structure. Neocortical rat atlases reveal discrepancies between authors, which may be attributable to differences in rat weight or strain. Using computer controlled measuring devices, Zilles & Wree (1985) defined neocortical areas of the rat, which are in close agreement with the commonly used stereotaxic atlas of Paxinos & Watson (1982). The frontal isocortical region is defined by three main regions, Fr1, Fr2 and Fr3, which border medially upon the anterior cingulate cortex, and laterally upon the agranular insular cortex, and may be differentiated from parietal areas by the lack of a prominent granular layer IV. The parietal cortex is divided into forelimb area (FL), hindlimb area (HL), Par1 and Par2, with appearance of FL and Par2 at more caudal levels. Par1 is the largest part of the parietal cortex and is bordered ventrocaudally by Par2 and dorsomedially by FL and HL. The occipital cortex is divided into two areas, Oc1 and Oc2, the former appearing at more caudal levels.

1.3 Cell types of the cortex

1.3.1 Pyramidal neurones

The shape of the cell body of the pyramidal neurones has similarities to the pyramids of ancient Egypt (Feldman, 1984). Pyramidal neurones of the neocortex are classified as small, medium or large, their size generally increasing with distance from the neocortical surface.

Additionally, there are "giant" pyramidal Betz cells, found in layer V of the motor areas in frontal cortex (Krieg, 1946), or pyramids of Meynert, found in layer V of the visual cortex (Parnavelas et al., 1983) with cell bodies as large as 100 μm in length. Pyramidal cells in layer IV are often ovoid, and are known as fusiform pyramidal cells. The length of their cell body is typically between 10-50 μm, a long axon (except for pyramidal cells of layer II), and apical dendrites which branch of in the direction of the surface of the neocortex (neocortical cells). The dendrites often bear a large number of spines which make synaptic contact with other neurones. A large proportion of the presynaptic elements in the neocortex are formed by axons which branch off from pyramidal neurones and which form collaterals within their area of origin (Peters, 1987).

Pyramidal cells form the major output systems of the neocortex, with those in layer II and III typically projecting ipsi- and contralaterally to other neocortical areas, and those of layer V projecting to subcortical sites.

1.3.2. Non-pyramidal neurones

According to staining characteristics and morphology, different types of non-pyramidal neurones can be identified in the neocortex (Warwick & Williams, 1973; Jones, 1975; Carpenter & Sutin, 1983). *Stellate nerve cells,* also called granule cells, is a class of cells which encompass a variety of specific cell types. They are generally small, and appear in variable amounts in all cortical laminae, except the most superficial (layer 1), but more abundantly in layers II and IV. Granule cells have a round soma which is drawn out at various angles by their dendrites, and a single, often relatively short axon. Another type of stellate cell, the *basket cell*, is horizontally extended, and has a short vertical axon which almost immediately divides into horizontal collaterals. These collaterals end in pronounced tufts, forming synaptic contacts with the somata and proximal parts of the dendrites of pyramidal cells. Basket cells form numerous axosomatic

synapses on the cell bodies of the pyramidal neurones. Other stellate cells include *neurogliaform stellate cells*. These are small neurones the dendrites of which arborise locally, and *stellate fusiform cells*, most commonly found in layers II, III and VI.

Other non-pyramidal cells included *horizontal cells* (of Cajal), which are only found in layer I of the neocortex. The axons of these neurones bifurcate and make synaptic contact with the dendrites of pyramidal neurones (Barr, 1974). The *cells of Martinotti* are present throughout the neocortex, except in the most superficial layers, as are *chandelier cells*. *Pleomorphic cells* are considered to be modified pyramidal cells, with variously shaped soma, whose axons enter the white matter. Their dendrites spread widely in the neocortex. Finally, *double bouquet cells* the soma of which can be found in layer II and III of the neocortex.

1.4 Connections of the rat cortex.

1.4.1 Neocortex

Cortical areas connect extensively both with subcortical structures and other areas of ipsi- and contralateral neocortex. The neocortex contains primary areas which are the main targets of sensory afferents (primary somatosensory, auditory and visual areas), and motor and association areas.

Connections of the frontal cortex of the rat brain, Fr1, Fr2 and Fr3 has been extensively studied (as reviewed, Zilles & Wree, 1985). These areas appear to be part of the motor cortex in the rat, with Fr1 and Fr3 probably forming the primary motor cortex (Donoghue & Wise, 1982). Besides afferents from the ventrolateral thalamic nucleus to Fr 1-3 and from the mediodorsal nucleus to Fr2, the frontal areas receive afferents from the thalamic nuclei (ventrolateral, ventromedial, parafascicular and intralaminar), geniculate nuclei, locus coeruleus and from almost all homolateral isocortical areas. Fr1 also receives afferents from the ipsilateral

and contralateral Fr2 and Par1 (via the corpus callosum), contralateral Fr1, ipsilateral FL and HL, and the basal forebrain nuclei. Contralateral projections of frontal regions are reciprocal. The frontal areas Fr1 and Fr3 form connections with the spinal cord, pontine nuclei and other subcortical structures. Homolateral and interhemispheric connections of the frontal areas originate both in layer III and V, though the homolateral association fibres are traditionally associated with layer III (MacClean, 1985).

1.5 The rat striatum

1.5.1. Morphology

The corpus striatum, so called because of its striped appearance, is one of the main components of the basal ganglia, and consists of the caudate putamen and the globus pallidus (Heimer et al., 1985). The caudate putamen alone may also be referred to as the striatum. The striatum extends ventrally to include large parts of the olfactory tubercle as well as the nucleus accumbens. Therefore, the most medioventral parts of the striatum, along with olfactory tubercle and nucleus accumbens are referred to as ventral striatum.

On the basis of their morphological characteristics several striatal cell types can be identified. Most important for identifying striatal neurones are the size of the soma and description of the spiny processes. More than 95 % of striatal neurones are medium sized (diameter 20-60 μm), whereas small neurones ($<$ 10 μm diameter) constitute a heterogenous and poorly described group of striatal cells (Chang et al., 1982). In the medium sized neurone group, the most commonly observed are the "medium spiny neurone" (Chang et al., 1982). These are the neurones which form projections to the globus pallidus and substantia nigra (Preston et al., 1980; Chang et al., 1981). Two types of large neurone (30-60 μm diameter) are found in the striatum. One group do not possess spines and have thick dendrites and those which

do possess somatic and dendritic spines (Heimer et al., 1985). The ventral striatum contains a similar distribution of morphologically distinct cell types.

1.5.2 Connections.

The dorsal and ventral striatum receives afferent projections from practically all neocortical and allocortical areas. Other major afferents come from the intralaminar thalamic nuclei and basolateral amygdaloid body. The entopeduncular nucleus, ventral pallidum and pars reticulata of the substantia nigra, in turn project to the thalamus, and, via collaterals of the same projections, to the brain stem pedunculopontine tegmental nucleus. Some neurones of the pars reticulata also send collaterals to the superior colliculus, thus providing a "striatal" input for the control of eye orienting and head orienting movements (Deniau & Chevalier, 1984; Evarts & Wise, 1984). Other pathways of the basal ganglia include reciprocal connections between the striatum, pallidonigral complex, subthalamic nucleus and pedunculopontine tegmental nucleus.

Connections between the striatum and the amygdaloid complex arise mainly from the basolateral amygdaloid complex, and reach almost all areas of the dorsal and ventral striatum (Heimer et al., 1985). Thalamostriatal projections originate in the intralaminar nuclei of the thalamus, and also in ventral, lateral and posterior thalamic nuclei. Other connections of the striatum include reciprocal connections to the substantia nigra, where the medium spiny neurones of the striatum innervate the reticulata, and receive innervation from the compacta neurones.

The principal efferent connection of the striatum is with the globus pallidus, entopeduncular nucleus and substantia nigra. The striatum is not thought to project directly to the neocortex. The primary efferents of the globus pallidus project to the subthalamic nuclei and the striatum, and also the entopeduncular nucleus and substantia nigra.

1.6 Transmitter systems of the cortex

1.6.1. Acetylcholine

Acetylcholine is synthesised in a reaction catalysed by Choline Acetyltransferase (ChAT): Acetyl CoA + choline results in ACh + CoA. Dale, (1938) was the first to postulate a central neurotransmitter role for acetylcholine (ACh). The parameters most used to determine the relative density of cholinergic neurones in tissue samples are the concentration of ACh, and the activity of ChAT (Fonnum, 1970; Hoover et al., 1978). Also, ChAT and acetylcholinesterase (AChE) can be used in histochemical studies on cholinergic innervation (Butcher, 1978; Armstrong et al., 1983).

The magnocellular nuclei located in the basal forebrain of the rat is considered to be the origin of cholinergic projections, because they stain both for ChAT and AChE. These nuclei include the nuclei of the medial septum, diagonal band, medial and lateral preoptic area, the basal nucleus , and the entopeduncular nucleus.

The nbM sends efferents to most areas of the neocortex (Bigl et al., 1982) and local cholinergic circuits may also exist, although this is still controversial.

The highest concentration of cholinergic terminals stemming from the nbM can be found in the upper layer IV and layer V. One consequence of this is that ACh may be involved in the modulation of activity of cortigofugal pathways (Eckenstein & Baughman, 1984).

1.6.2 5-HT

5-HT is found in many cells that are not neurones, such as platelets, mast cells, and the enterochromaffin cells (as reviewed in: Cooper et al., 1991). Nevertheless, because 5-HT cannot

cross the blood brain barrier brain cells must synthesise their own. The first step in this process is the uptake of the amino acid tryptophan, which is the primary substrate for the synthesis. Tryptophan is hydroxylated at the 5 position to form 5-Hydroxytryptophan. This reaction is catalysed by the enzyme tryptophan hydroxylase. Once synthesised, 5-Hydroxytryptophan is almost immediately decarboxylated to yield 5-HT (Cooper et al., 1991).

After being released from the nerve terminal, 5-HT is deaminated by monoamine oxidase. The product of this reaction, 5-hydroxyindoleacetaldehyde can be further oxidized to 5-hydroxyindoleacetic acid (5-HIAA) or reduced to 5-hydroxytryptophol, depending on the NAD^+/NADH ratio in the tissue (Cooper et al., 1991).

The greatest proportion of serotonergic innervation to the cortex stems from the raphe system, a system which shows little variation across species (Petrovicky, 1980). The raphe nuclei are located in the brainstem and are subdivided by most into seven regions, of which the dorsal raphe nucleus (DRN) is the largest. Serotonergic projections coming from other areas in the brain, outside the raphe complex include those of the hypothalamus, the mesencephalic, pontine and medullary reticular formation and the interpeduncular complex (Törk, 1985). The ascending projections of the raphe nuclei go via two main routes, which join at the level of the hypothalamus in the medial forebrain bundle.

The existence of a serotonergic innervation of the cortex was first demonstrated by histofluorescence (Dahlström & Fuxe, 1964; Ungerstedt, 1971), but the full extent of this innervation was recognised only later with the introduction of the retrograde tracing techniques. It is now well established that there is a distinct projection from the dorsal and median raphe nuclei to the cortex (Kellar et al., 1977; Segal, 1977; Arikuni & Ban, 1978; Bentivoglio et al., 1978; Divac & Kosmal, 1978; Gerfen & Clavier, 1979; Törk et al., 1979). The histofluorescence and immunocytochemical investigations clearly established that the serotonergic projections from the dorsal and median raphe nuclei reach the cortex via several routes. Initially, all fibres run in the ventral serotonergic bundle which is part of the medial

forebrain bundle, but then a group of them moves laterally from the lateral hypothalamus to the amygdala-entorhinal cortex area. Branches from this lateral bundle ascend in the external capsule to the lateral cortex. Axons that continue in the medial forebrain bundle enter the medial and posterior cortex through the supracallosal cingulum bundle; if they continue rostrally, they reach the frontal cortex (Ungerstedt, 1971; Tohyama et al., 1980; Lidov & Molliver, 1982).

Detailed characterisation of the 5-HT innervation of the rat cerebral cortex (Lidov et al., 1980; Lidov & Molliver, 1982) has revealed that the density of 5-HT axons is highest in the superficial layers of the cortex, although 5-HT containing axons are found through the whole thickness of the cortex (Lamour et al., 1983). Most of the 5-HT axonal varicosities in the superficial layers do not appear to be involved in any synaptic contact (Descarries et al., 1975). In the lateral neocortex the orientation of the 5-HT fibres varies with the layers. In layer I most fibres run parallel to the pial surface, while in layers II and III, the axons run in parallel bundles normal to the pial surface. The fibres in layers IV and V are extremely thin and highly tortuous, while in layer VI they are arranged parallel to the pial surface.

1.6.3 GABA

Synthesized in 1883, γ-aminobutyric acid (GABA) was known for many years as a product of microbial and plant metabolism. Not until the 1950's, however, was GABA identified as a normal constituent of the mammalian central nervous system. Horton, (1989) estimated that 30-40 % of all the synapses in Central Nervous System utilise GABA as a neurotransmitter. The vast majority of GABA-ergic neurones are short interneurones, mediating mostly local inhibitory control of the innervation targets (Jones, 1986). Three primary enzymes are involved in the metabolism of GABA before its entry into the Krebs cycle. GABA is formed by the α-decarboxylation of L-glutamic acid, a reaction catalysed by glutamic acid decarboxylase. GABA is intimately related to the oxidative metabolism of carbohydrates in the central nervous

regenerating glutamate, and its entry into the Krebs cycle as succinic acid via the oxidation of succinic semialdehyde by succinic semialdehyde dehydrogenase. The visualisation of GABA-ergic interneurones is possible with immunocytochemical markers. A much used marker is the enzyme glutamic acid decarboxylase

Studies using this marker reveal large populations of these neurones in the neocortex (Hendry & Jones, 1986).

1.6.4 Noradrenaline

Noradrenaline belongs to a class of compounds known as catecholamines. The term "catecholamine" refers generically to all organic compounds that contain a catechol nucleus (a benzene ring with two adjacent hydroxyl substituents) and an amine group. In practice, the term comprises dihydroxyphenylethyl amine (dopamine) and its metabolic products noradrenaline and adrenaline. The catecholamines are formed in brain, chromaffin cells, sympathetic nerves and sympathetic ganglia from their amino acid precursor tyrosine by a sequence of enzymatic steps. The first step involves the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA). The ultimate formation of noradrenaline, adrenaline or dopamine depends on the availability of phenylethanolamine-N-methyl transferase and dopamine-ß-hydroxylase. The first enzyme in the biosynthetic pathway, tyrosine hydroxylase is a unique constituent of catecholamine-containing neurons and chromaffin cells. The enzyme is stereospecific, requires molecular O_2, Fe^{2+} and a tetrahydropteridine cofactor and oxidizes the naturally occurring amino acids, L-tyrosine to yield DOPA, and to a smaller extent, L-phenylalanine. The second enzyme involved in catecholamine biosynthesis is DOPA-decarboxylase which removes carboxyl groups from all naturally occurring aromatic L-amino acids. This enzymatic step results in the formation of dopamine. The third enzymatic step in the formation of noradrenaline is that catalysed by dopamine-ß-hydroxylase, a mixed function oxidase. It requires molecular oxygen and utilises

ascorbic acid as a cofactor. In the adrenal medulla noradrenaline is N-methylated by the enzyme phenylethanolamine-N-methyl transferase to form adrenaline. This enzyme is largely restricted to the adrenal medulla, although low levels of activity have been reported in heart and mammalian brain (Cooper et al., 1991).

The major mammalian enzymes of importance in the metabolic degradation of catecholamines are monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). MAO converts catecholamines to their corresponding aldehydes, ultimately resulting in the formation of 3-methoxy-4-hydroxy-phenylglycol. The reaction catalysed by catechol-O-methyl transferase ultimately results in the formation of homovanillic acid.

The major source of noradrenergic innervation to the cortex arises in the locus coeruleus (Swanson, 1976). This input exhibits a certain degree of topography: more anterior locus coeruleus cells project to anterior neocortical regions.

1.7 Receptors of the cortex.

1.7.1 Muscarinic receptors.

Muscarinic receptors are widely found throughout the mammalian brain (Yamamura et al., 1974; Yamamura & Snyder, 1974; Levey et al., 1991). Many studies have centered on their role in cognitive function, as small doses of the antagonist scopolamine was shown to induce confusion and amnesia (Drachman & Leavitt, 1974).

The use of molecular biology techniques has advanced the understanding of the subdivision of the muscarinic type cholinergic receptors. Thus, earlier studies which subdivided muscarinic receptors into M_1 and non-M_1 (Hammer et al., 1980) have given way to a wider classification based on the cloning of m_1-m_5 genes (Bonner et al., 1987; Bonner et al., 1988) which give rise to M_1-M_5 receptors. However, the pharmacological distinction between these subtypes is often unclear (Buckley et al., 1988), and only three distinct physiological subtypes have been pharmacologically classified.

The Muscarinic receptors are classified as G-protein-coupled receptors, which activate a second messenger signal transduction mechanism (Bonner et al., 1987; Loring & Zigmond, 1988). Activation of the M_1 receptor stimulates PLC activity (Fisher & Agranoff, 1987) via the G-protein G_q. PLC cleaves inositol-containing phospholipids (phosphoinositides), generating the primary second messengers inositol triphosphate (IP_3) and DAG, which increase internal Ca^{2+} concentration, and activate PKC, respectively.

The M_2 receptor is linked to the G-protein G_i, which inhibits the enzyme adenylyl cyclase, reducing the production of cyclic-adenosyl monophosphate (cAMP). It is thought that the postsynaptic excitation of pyramidal neurones is mediated by the M_1 receptor (McCormick & Prince, 1985; McCormick & Williamson, 1989), while the M_2 receptors inhibit transmitter

release by a presynaptic mechanism (reviewed by McCormick, 1992), and may stimulate GABA-ergic interneurones to reduce pyramidal cell excitability indirectly (McCormick & Prince, 1985; McCormick & Prince, 1986).

1.7.2 Nicotinic receptors

Traditionally, nicotinic acetylcholine receptors have been categorised in two classes: muscle type and neuronal type. The irreversible antagonist, α-bungarotoxin is selective for the muscle type, while k-bungarotoxin has been utilised to label neuronal nicotinic receptors (Loring & Zigmond, 1988). Neuronal nicotinic receptors are widely distributed throughout the brain (Clarke et al., 1985), and are thought to be involved in pre- and postsynaptic actions of ACh. The role of nicotinic receptors in cognitive function has been extensively studied (as reviewed: Levin, 1992). Recently, seven genes that encode for neuronal nicotinic receptors have been identified (Heinemann et al., 1991b), and it is likely that these genes code for a number of different receptor subunits, composed from different gene product combinations, expressed in mammalian brain. This underlies the difference between muscle and neuronal nicotinic receptors; genes encoding for muscle receptors yield six distinct subunits , of which five (α1, ß1, γ, δ and ϵ) make the pentameric oligomer forming the final receptor. Neuronal receptors, which are also pentamers, are composed from combinations of only two subunit types (α2-5 and ß2-4). It is thought that the α subunit contains the high affinity nicotine binding site. As yet, functional differences between physiologically expressed neuronal types of nicotinic receptor are unknown, although their distribution may by visualised in the brain by *in -situ* hybridisation histochemistry (Wada et al., 1989).

Activation of the neuronal nicotinic receptor opens a cation channel which gates Na^+ and depolarises the associated neurone. The depolarisation may be of short duration and amplitude, giving rise to an excitatory postsynaptic potential , which may summate with excitatory

postsynaptic potentials produced from activation of other excitatory receptors, although nicotinic receptor activation alone is capable of producing an action potential.

1.7.3 5-HT receptors

As with other receptors described, there are a number of different 5-HT receptor subtypes, first described by Gaddum & Picarelli (1957). The classification into "D" and "M" types was changed to a classification in to 1,2 and 3 (Bradley et al., 1986), although new subtypes of the 5-HT_1 receptor are currently being classified (Beer et al., 1993), and include new subtypes of the 5-HT_1 receptors, and 5-HT_{4-7} (Hoyer et al., 1994). In the CNS, ligand binding as well as molecular biology has been used to classify 5-HT receptors, which, on this basis may be classified into 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 subtypes (Peroutka & Snyder, 1979; Kilpatrick et al., 1987; Radja et al., 1991).

The 5-HT_1 receptor may be further subdivided into 5-HT_{1A} 5-HT_{1B} 5-HT_{1C} (now reclassified as 5-HT_{2C}), 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} (Hoyer et al., 1994).The 5-HT_{1B} is considered to be a species isoform found in the rat and the mouse, and is equivalent to the 5-HT_{1D} receptor (Radja et al., 1991).

The 5-HT_2 receptor has also been cloned, and other than the renamed 5-HT_{1C} receptor, two other subtypes have been identified: the 5-HT_{2A} receptor and the incompletely characterised 5-HT_{2B} receptor (Schmuck et al., 1994).

5-HT_1 and 5-HT_2 receptors are members of the seven transmembrane spanning region, G-protein linked receptor superfamily. In contrast, the 5-HT_3 receptor is strikingly different in that its properties are typical of a 5-HT gated ion channel (Derkach et al., 1989). Members of the 5-HT_1 family are coupled via G to adenylyl cyclase, and thus receptor activation reduces intracellular concentrations of cAMP.

Studies performed by Pazos & Palacios (1985), who used three different ligands ([^{3}H]5-HT; [^{3}H]8-Hydroxy-dipropylaminotetraline and [^{3}H] Lysergic acid diethylamide) to investigate the presence and distribution of 5-HT_{1A} receptors in rodent and mammalian brain show considerable differences between species. In the rat the hippocampal formation was found to be very enriched in these receptors, with the dentate gyrus showing the highest density of these sites throughout the brain. The different fields of the hippocampus (CA1-CA4) also represent intermediate to high levels of binding, with the oriens layer being the most enriched. The dorsal subiculum, an area containing a very high density of 5-HT_{1B} receptors, was poor in 5-HT_{1A} receptors. The septal region also exhibited a very high level of 5-HT_{1A} labelling. Other structures showing high to intermediate densities of autoradiographic grains in the rat brain included the entorhinal cortex, some nuclei of the amygdala, the ventromedial hypothalamic nucleus and the raphe nuclei, primarily the dorsal raphe nucleus. The distribution of 5-HT_{1A} receptors in the different areas of the neocortex showed relatively low amounts of binding over the external layers, while the internal ones (V-VI) contained high concentrations of binding sites. Other regions, such as basal ganglia, thalamus, brain-stem (with the exception of the raphe nuclei) and spinal cord were, in general, poor in 5-HT_{1A} binding sites.

Studies on the distribution of 5-HT_{1A} receptors in the human brain (Hoyer et al., 1986a; Pazos et al., 1986) show a heterogenous distribution of these receptors. Similar to the rat, the hippocampal complex was the area most enriched in 5-HT_{1A} binding sites throughout the human brain. The CA1 field, at the different layers analysed, contained a very high density of autoradiographic grains. The dentate gyrus and the subiculum also presented high levels of specific binding. The cortical areas were also rich in 5-HT_{1A} receptors. However, the distribution of these sites were not homogenous. Thus, while high levels of specific binding were found in the external layers, especially in layer II. the amount of binding in the internal ones (IV-VI) was lower. An important species difference (between rodents and human cortex) in localisation of 5-HT_{1A} receptors was observed. A study on neurosurgical samples of neocortex

by Francis et al., (1993b) revealed that the highest number of $5HT_{1A}$ receptors were present in the superficial layers. Also the raphe nuclei were rich in 5-HT_{1A} binding sites. The nucleus raphe centralis (pars annularis), the nucleus raphe dorsalis (pars supratrochlearis) and the nucleus raphe linearis (at the mesencephalic level) were the most enriched in these receptors.

The electrophysiological actions of the 5-HT_{1A} receptor have been extensively investigated in both rat (Beck & Choi, 1991; Andrade, 1992) and human brain preparations (McCormick & Williamson, 1989). Activation of these receptors increases potassium conductance and inhibits cell firing. Postsynaptic sites mediate inhibition of pyramidal neurones of the cortex and hippocampus, while 5-HT_{1A} receptors localised on the cell bodies of DRN neurones regulate activity of these cells (Radja et al., 1992; Blier et al., 1993).

1.8 Alzheimer's disease- general overview

1.8.1 History, incidence and symptoms.

Alois Alzheimer was the first to describe, in an autopsy report, the neuropathology of the disease which later was called Alzheimer's disease (AD) after him, although Esquirol had earlier described the clinical symptoms (Esquirol, 1838) In his report Alzheimer described the neuropathological findings in the *post mortem* brain of a 55 year old woman who had suffered from progressive dementia (Alzheimer, 1907). In this report he described many abnormal nerve cells containing tangles and fibres. In addition he noted a reduction in the number of neurones.

All over the world AD is causing enormous medical and social problems, problems which can only increase as people live longer. A recent study (Hofman et al., 1991) suggests that in Europe the risk of getting dementia increases exponentially with age. For the five-year age groups from 60 to 94 the overall European prevalence increases from 1.0, 1.4, 4.1, 5.7 to 13.0, 21.6 and 32.2 % respectively. Of all the dementia cases, AD is the major single cause,

regardless of the age of onset. Of dementing disorders, AD accounts for approximately 40 % observed in the presenium (Marsden & Harrison, 1972) and 50 % observed in senile years (Tomlinson, 1977).

AD is a disorder with insidious onset, which shows slow but relentless progression. Initial symptoms of forgetfulness (forgetting where one has placed familiar objects and/or forgetting names) develop into more clearcut deficits (e.g. getting lost when travelling to an unfamiliar place; word and name finding deficit; problems with retaining material; concentration deficits). Later on in the disease, patients can no longer survive without some assistance. Frequently some disorientation to time and/or place becomes evident. The later stages of the disease is characterised by loss of all verbal abilities. Frequently there is no speech at all, patients suffer from incontinence of urine, they require assistance in toileting and feeding and lose basic psychomotor skills, e.g. ability to walk. In addition, personality and emotional changes occur, for example depressive symptoms, delusional behaviour, obsessive symptoms, anxiety, agitation and aggression. Dementia symptoms of this sort are associated with a sharp decrease in life expectancy; the disease course lasts on average 6 years (Sulkava et al., 1983).

Although it is from a practical point of view convenient to focus on one aetiological factor at the time, it seems likely that the disease is caused by a complex interplay of multiple advents in the patient's brain.

1.8.2 Diagnosis.

It is difficult to diagnose AD in the living patient with absolute certainty. Diagnosis of AD is assumed to exist when other disorders which can cause dementia are ruled out. These include toxic, nutritional, infectious, endocrinological, neoplastic, traumatic and circulatory diseases. Symptoms of the disease comprise cognitive impairment (American Psychiatric Association, 1980), although non-cognitive symptoms (e.g. aggression, wandering, incontinence and

depression) can also be a major feature. The diagnosis can only be confirmed by the finding, in *post-mortem* neuropathological examination, of the histological hallmarks of the disease: senile plaques and neurofibrillar tangles (McKhann et al., 1984).

The two most common tests of mental status are verbal information concentration orientation test (Blessed et al., 1968), which assesses recent and past memory, and the mini-mental state evaluation test (MMSE), (Folstein et al., 1975), which assesses drawing, writing and language skills.

1.9 Alzheimer's disease- neuropathology.

1.9.1 Overview

The *post-mortem* examination of the brain of suspected sufferers of AD normally reveals characteristic macroscopic and microscopic features (Tomlinson & Corsellis, 1984). The brain is smaller than a normal control, weighing 60-80 % of normal, which is caused by the loss of both grey and white matter. The gyri are reduced in width and the sulci wider. Some areas are more affected than others. Temporal and parietal areas are particularly affected, with less frontal lobe atrophy. Areas of association cortex are heavily involved, whereas the primary sensory areas are relatively spared (Brun, 1983; Tomlinson & Corsellis, 1984; Esiri et al., 1986; Esiri et al., 1990). It seems likely that the reductions in the width of the affected cortical areas are the result of loss of cortical pyramidal neurones. Loss of neurones forming association pathways (as reviewed, Hof & Morrison, 1994) in circumscribed (temporal and parietal) neocortical and hippocampal (CA1) areas is primarily due to a disappearance of pyramidal neurones (Terry et al., 1991). The remaining neurones may develop tangles, (intracellular deposits of fibrous material) which are especially notable in layer III and V of the cortex (Pearson et al., 1985; Lewis et al., 1987), neurones in these layers of the cortex form the origin of the majority of

efferent pathways (Jones, 1984). That neuronal loss and the effect of tangle formation may underlie the cognitive deficits observed in AD, is supported by findings of several studies where significant correlations between synapse and cell loss in these areas and ratings of dementia have been described (Neary et al., 1986; DeKosky & Scheff, 1990; Terry et al., 1991). In addition to the intracellular deposition of fibrilloid material, extracellular deposits, mainly consisting of a 39-42 amino acid peptide called ß-amyloid are present in the brain of patients suffering from AD.

1.9.2 Plaques.

The senile plaque is a complex structure (see for review: Selkoe, 1994) and the precise way in which it develops over time is imperfectly understood. So called "classical" neuritic plaques (also referred to as senile plaque), consists of a deposit of extracellular filaments, the main constituent of which has been found to be ß-amyloid (also termed ßA4). The plaque is normally surrounded by variable numbers of dystrophic neurites, both originating from degenerated axones and dendrites. Such plaques may also contain activated microglial cells which normally are located next to the amyloid core (Wisniewski et al., 1989), as well as reactive astrocytes which are normally located further away from the core. Amyloid is a generic term used to describe a group of chemically heterogenous proteins found in a number of different tissues and diseases. Amyloid in its deposited form gives rise to the congo red birefringence and proteolysis resistant characteristics of the classical plaque. Glenner and Wong were the first to report the amino acid sequence of senile plaque amyloid. They observed great similarities between amyloid found in the brain parenchyma and that isolated from meningeal blood vessels (Glenner & Wong, 1984).

In the Alzheimer brain many classical plaques can be found in the hippocampus, amygdala, entorhinal cortex and neocortical association areas, but investigations using highly sensitive

antibodies to ßA4 have revealed that they are a minority of all neuronal ßA4 deposits In most AD brains deposits of ßA4 referred to as "diffuse" or "pre-amyloid" plaques can be observed (e.g. Tagliavini et al., 1988). When examined with the electron microscope, they display few if any structurally altered neurites, astrocytes or microglial cells and their ßA4 immunoreactivity is not explained by amyloid filaments, which are very sparse or absent (Verga et al., 1989; Yamaguchi et al.,1990). The precise structural and biochemical form of ßA4 found in diffuse plaques remains an unsettled issue. An interesting finding is that diffuse plaques, are also found in the brains of young Down's syndrome patients, while dystrophic neurites or tangles are not notable in these brains. It is therefore assumed by some that the diffuse plaques evolves into senile plaques in the course of AD (as reviewed, Selkoe, 1994).

In addition, a variety of normally soluble molecules can accumulate in the plaque. For example α_1-antichymotrypsin (Abraham et al., 1988), several components of the classical complement cascade (Eikelenboom & Stam 1982, Rozemuller et al., 1990), heparan sulfate proteoglycan (Snow et al., 1988) and the serum amyloid P component (Kalaria et al., 1991)

1.9.3 Neurofibrillary Tangles

As opposed to the extraneuronally located senile plaque, the *post-mortem* AD brain is also characterised by intraneuronal deposits. These are commonly called neurofibrillary tangles (NFT's). A number of different types have been identified in the past. NFT's are found in large pyramidal neurones and in apical dendrites. They are found in distal dendrites as neuropil threads and in the abnormal neurites that are associated with some amyloid plaques (neuritic plaques) (for review see: Goedert, 1993). All three lesions contain paired helical filaments (PHF) as their major component (Kidd, 1963; Terry, 1963) and a number of straight filaments as a minor fibrous component. The development of the NFT's follows a fairly consistent pattern. Certain cell types and cellular layers and brain regions are affected with little individual

variation. This phenomenon has recently been used to define six neuropathological stages of AD (Braak & Braak, 1991). The very first neurones in the brain to develop NFT's are located in layer pre-α of the trans-entorhinal cortex, thus defining stage I. In stage II this region is more severely affected. In addition the pre-α region of the entorhinal cortex begins to show NFT's. Patients in this stage of the disease are clinically still unimpaired. Impairment of cognition becomes apparent in stages III and IV, when NFT's become apparent in the pre-α layers of both entorhinal and transentorhinal regions. Stage IV also sees the appearance of NFT's in the deep pre-α layers. Pathology during stages III and IV spreads to layer I of the Ammon's horn of the hippocampus and in a number of subcortical nuclei, such as the basal forebrain magnocellular nuclei and the anterodorsal thalamic nucleus. In the last stages of the disease (V and VI), a massive development of NFT's in isocortical association areas can be observed. The stereotyped nature of the temporal and spatial development of NFT's contrasts with the development of the ßA4 deposits. It is therefore not surprising that the amount of NFT's correlates much better with both the degree of dementia and neocortical synapse number than does senile plaque number (Terry et al., 1991). It may not be all that surprising that a structure which develops intraneuronally will show better correlations with other disappearing neuronal structures than a diffuse extraneuronal structure, which will invariably show much more variability in the total amount of neurones involved.

Current evidence suggests that PHF are made entirely of the microtubule associated protein, tau, in an abnormally phosphorylated state. The PHF is made out of two strands of subunits which twist around each other forming a helix. PHF can be isolated both from tangle fragments or more dispersed filaments (Wischik et al., 1988; Greenberg & Davies, 1990). Both types of PHF have tau epitopes in common, but differ in their solubility in strong denaturing agents, and sensitivity to proteases (Goedert et al., 1992; Goedert, 1993). Straight filaments also contain PHF, which also share tau epitopes with the former types (Goedert, 1993). Tau containing PHF is therefore a common feature of neurofibrillary lesions.

1.9.4. Amyloid precursor protein.

It is generally accepted that the main constituent of the amyloid plaques is the 4kDa amyloid protein (ßA4) (Glenner & Wong, 1984), which is a proteolytic product of a precursor protein of much larger size, the amyloid precursor protein (APP).

APP resembles a receptor in that it has a membrane-spanning region, a large ectodomain and a short cytoplasmic tail (Kang et al., 1987). Different isoforms of APP result from alternative splicing of three exons in a single gene (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; König et al., 1992). One of these, exon 7, codes for a 56 residue serine protease inhibitor of the Kunitz-type (KPI). Another, exon 15, encodes a small region near the transmembrane region.

APP-related proteins which are derived from other genes, the amyloid precursor-like proteins APLP1 and APLP2, are very similar to APP, but differ with that peptide in the juxta-membranous regions of the exoplasmic and transmembrane domains, where the ßA4 sequence is located. APLP1 and APLP2 are therefore unable to produce ßA4 or ßA4-containing fragments.

ß-A4 is partly located extracellularly and partly in the transmembrane portion of APP. Some APP is released by an enzyme dubbed α-secretase which cleaves within the ßA4 domain (Esch et al., 1990) to release secreted APP (APP_s), and precludes the release of intact ßA4. Another portion of APP_s is released by a ß-secretase, which cleaves near the NH_2-terminus of ßA4 (Seubert et al., 1993) and produces COOH-terminal fragments which contain the whole ßA4 domain. The finding of intact ßA4 and other fragments of the ßA4 domain (p3) in the extracellular compartment (Haass et al., 1992; Seubert et al., 1992) suggests that other proteolytic activities exist under normal conditions, which can generate the COOH-terminus of ßA4, releasing the transmembrane domain from the lipid bilayer, In addition to these secretory

mechanisms, there are storage mechanisms in which the regulated release of APP_s occurs. This is best exemplified by the storage of APP_s in the α-granules of platelets (van Nostrand et al., 1990).

Early studies showed that cultured neuronal cells as well as transfected cell-lines which over-expressed APP releases 105-125 kDa APP_s isoforms into their culture medium (Schubert et al., 1989; Weidemann et al., 1989). Furthermore, APP_s lacking the COOH-terminus of the full-length integral forms were found and extracted from human CSF and brain tissue (Palmert et al., 1989). These forms existed both in AD and in normal control subjects. Thus APP secretion appeared to be a normal event, and it was predicted to result from a cleavage in the vicinity of ßA4. The demonstration that the protease nexin II, a soluble protease inhibitor involved in cell growth regulation, was identical to a secreted isoform of APP with the KPI domain (Oltersdorf et al., 1989; van Nostrand et al., 1989) brought additional evidence for the process being a normal secretion event.

Using cell lines transfected with several constructs of APP_{770} that contained the whole or parts of ßA4, Sisodia et al., (1990) showed that APP secretion was membrane-associated and occurred within the exoplasmic portion of ßA4.

Studies with transfected cell lines have shown that substitutions at or immediately near the α-secretase site did not impede secretion (Zhong et al., 1994; Maruyama et al., 1991; Wang et al., 1991; de Strooper et al., 1993). These studies indicate that α-secretase is not sequence specific, but that it recognizes an α-helix structure and cleaves at a certain distance from the membrane, independent of amino acid sequence (Maruyama et al., 1991). One interpretation of these studies is therefore that α-secretase might cover several proteases which release APP. In addition to the variability of α-secretase cleavage sites there is now ample evidence for an alternative secretion mechanism which releases the ßA4 NH_2 terminus. For example, Seubert et al., (1993) demonstrated in human brain and cerebrospinal fluid (CSF) the COOH-terminal heterogeneity of secreted APP. Using mixed-brain cultures they showed with specific antibodies

that some forms of secreted APP ended just before ßA4 NH_2 terminus and concluded that these should be the products of a "ß-secretase". This result complemented other studies which showed the release of intact ßA4 in cerebrospinal fluid (CSF) and in plasma as well as in culture media (Haass et al., 1992; Seubert et al., 1992; Shoji et al., 1992). Taken together these results suggest an alternative secretory pathway which would lead to the release of soluble ßA4 but could also lead to the release of larger peptides, less soluble and potentially more amyloidogenic.

An important advance related to ß-secretase specificity came when a pathogenic mutation of APP was found in a Swedish family. Mullan et al., (1992) found a double mutation in the APP gene at residues -2 and -1 of ßA4, just before the ßA4 NH_2 terminus (Lys 594-Met 595 of APP_{695} were changed to Asn 594-Leu 595 in the mutation). The direct influence of this double mutation on the ß-secretion of APP was further demonstrated in transfected cell systems (Citron et al., 1992; Cai et al., 1993; Felsenstein et al., 1994), showing a 6-8-fold increase of ßA4 secretion by the cells transfected with the APP Swedish mutation compared with those transfected with the wild type of APP.

Much work has focused on where in the cell processing of APP occurs. α-Secretase cleavage requires association with the membrane since constructs in which the cytoplasmic domain plus a part of the transmembrane domain are deleted and replaced with substance P are excreted but not cleaved (Sisodia, 1992). Secreted APP has been found intracellularly (Sambumarti et al., 1992) and there is some evidence that secretase cleavage occurs after sulfation in the late-Golgi compartment (de Strooper et al., 1993; Kuentzel et al., 1993). Inhibiting the protease activities of the lysosomal/endosomal system does not diminish secretion, indicating that release of APP does not take place in this compartment (de Strooper et al., 1993).

Several functional domains have been characterised within the APP cytoplasmic region, including a putative binding site for the G_o GTP-binding protein (Nishimoto et al., 1993), several phosphorylation sites (Gandy et al., 1988; Suzuki et al., 1994) and a signal for reinternalisation (Nordstedt et al., 1993).

Several functions of APP have been identified. The protein has a role in cell-cell interactions, can be neuroprotective and increases synapse formation.

A third possible secretase activity, called the γ-secretase is thought to free the ßA4-COOH-terminus.

In conclusion, APP secretion appears to be a complex process. The use of different cell types and various transfection protocols illustrates the different approaches to investigating the secretion pathways.

1.9.5 Evidence for toxicity of ßA4.

The main constituent of senile plaques, one of the histological hallmarks of AD is ßA4. In recent years a lot of effort has focused on the possibility that this peptide may be neurotoxic. Many *in vitro* cell cultures suggest that under certain circumstances ßA4 can indeed be neurotoxic. It has been reported that the age (Yankner et al., 1989), cell density and source of the cultured neurones (Mattson et al., 1992), as well as the soluble (Yankner et al. ,1990) or aggregated state of the peptide are important factors in mediating the *in vitro* effects of ßA4 (see Neurobiol. Aging 13, 1992, whole issue on ßA4 toxicity). The peptide can produce both neurotrophic and neurotoxic effects in cultured hippocampal neurones. When applied on the first day of culture, synthetic ßA4 (1-40) promotes neurite branching and axonal elongation, but when applied to cultured hippocampal neurones at day 4 of the culture, ßA4 is neurotoxic (Yankner et al., 1990). This suggests that the aggregation state of the peptide is the main determinant of toxicity. It remains unknown whether the effect of in vitro neurotoxicity of ßA4 is direct or indirect. For example Yankner et al., (1990) suggest that ßA4 is directly toxic to cultured neurones. However, other investigators, using cultured mouse and human cortical neurones, have demonstrated that while ßA4 is not by itself neurotoxic, it increases the vulnerability of these neurones to other challenges. For example, Koh et al.,(1990) reported that cultured mouse

cortical neurones that had been pretreated for 2 days with ßA4 (1-42) were more vulnerable to glutamate neurotoxicity than were control cultures. Subsequently Mattson et al., (1992) reported that pretreatment (2-4 days with ßA4 1-38 or ßA4 25-35) also rendered human cortical neurones vulnerable to excitotoxicity. This effect, however, required days of incubation with ßA4, a time course which is atypical for receptor-mediated Ca^{2+}- influx but may reflect altered cell permeability secondary to chronic ßA4-induced membrane damage. Thus it remains to be determined whether increased neuronal Ca^{2+} is a primary cause or secondary effect of ßA4-induced membrane damage. The dependence of neurotoxicity on the physical state of the ßA4 peptide raises the possibility that the mechanism of toxicity may involve direct physical disruption of the neuronal architecture.

Kowall et al., (1991) have reported that the effects of ßA4 may be mediated through specific receptors, since both the neurotrophic and neurotoxic effects of ßA4 in cultured hippocampal neurones *in vitro* were blocked by substance P and other tachykinins.

The exact physiological role, if any, of ßA4, and its contribution to the development of the pathology of AD remains to be elucidated.

1.10 Neurotransmitters in Alzheimer's disease.

1.10.1 Acetylcholine

One of the most consistently described deficits in AD brains is that of the cholinergic system, first described by Bowen et al. (1976), closely followed and corroborated by other groups (Davies & Maloney, 1976; Perry et al., 1977a; Perry et al., 1977b; Davies, 1979).

Neuropathological studies confirm that there is usually a considerable loss of cholinergic neurones of the nucleus basalis of Meynert (nbM) (Whitehouse et al., 1982; Nagai et al., 1983).

The loss of cholinergic markers has been correlated both with the severity of dementia (Francis et al., 1985; Neary et al., 1986), and neuropathological changes (Mountjoy et al., 1984).

Studies of cholinoreceptor populations are discrepant, with reports of decreased, increased or unchanged numbers of muscarinic receptors (Whitehouse et al., 1987; Giacobini, 1990; Perry et al., 1990; Flynn et al., 1995), although the M_2 receptor, thought to represent a presynaptic autoreceptor, is more consistently reduced (Mash et al., 1985; Nordberg et al., 1992; Svensson et al., 1992). *In-situ* hybridisation histochemistry for m_1 mRNA also revealed both increases (Harrison et al., 1991) and decreases (Wang et al., 1992). The nicotinic receptor is reported to be reduced in most studies (Giacobini, 1990), and this may reflect loss of both pre- and postsynaptic sites

1.10.2 Noradrenaline

A number of studies describe loss of locus coeruleus cell bodies in AD (Mann et al., 1980; Bondareff et al., 1981). There is also a corresponding loss of noradrenalin (NA) in some neocortical areas (Cross et al., 1981; Francis et al., 1985), although the concentration of the NA metabolite 3-methoxy-4-hydroxyphenethyleneglycol has been reported to be elevated (Gottfries et al., 1983) or unaltered (Palmer, 1987c). Studies of biopsy tissue from AD patients on NA concentration, uptake and release revealed reduced concentration and uptake of NA in the temporal lobe, although release in the frontal lobe was unaffected (Palmer, 1987c). Deficits in NA do not appear to correlate with dementia, although they may contribute to the non-cognitive symptoms (Fowler et al., 1992; Palmer et al., 1988).

Postsynaptic α and ß adrenoreceptors are unaffected in most studies of autopsy AD material , although there is some evidence of regional increases in binding to ß receptors in hippocampus and cortex (as reviewed: Young & Penney, 1994; Cowburn et al., 1989; Francis et al., 1992a). α_2 adrenergic receptors are thought to exist presynaptically on the terminals of noradrenergic

neurones projecting from the locus coeruleus. Loss of high affinity α_2 receptor binding has been reported in *post-mortem* tissue from frontal cortex and hippocampus of AD brain (Pascual et al., 1992).

1.10.3 Serotonin

The situation regarding presynaptic serotonergic markers is complex. Biochemical estimates of 5HT-containing neurones in *post-mortem* samples have relied mostly on determining concentrations of 5HT and its major metabolite 5HIAA. Their concentrations may be reduced in the neocortex of AD patients. However, even in autopsy AD patients half of many cortical areas showed no evidence of a selective reduction in presynaptic 5HT activity (Palmer et al., 1988). Confounding factors cloud the issue even more. For example behavioural symptoms, like depression, may influence serotonin markers in cortical tissue. Indeed a recent study focusing on non-institutionalised patients suffering from AD observed no decrease in the presynaptic serotonergic markers (5HT-uptake site, 5HT and 5HIAA concentration).

Most studies support a decrease in forebrain serotonin (5-HT) receptors, with reductions in binding to 5-HT_1 (Cross et al., 1984a, Cross et al., 1984b) 5-HT_{1A} receptors (Middlemiss et al., 1986b; Bowen et al., 1989) and 5-HT_{2A} (Cross et al., 1984a.) receptors reported. These reductions in 5-HT_{2A} binding are considered to reflect loss of neocortical interneurones that have been found to be reduced in a number of neocortical brain areas (Procter et al., 1988a; Bowen et al., 1989a).

1.10.4 γ-Aminobutyric acid (GABA).

Although the assessment of the activity and integrity of GABA containing interneurones in *post-mortem* tissue may be affected by agonal state, several reports indicate loss of markers of

these neurones from the cortex of AD patients (Rossor et al., 1982; Ellison et al., 1986). However, a study of *ante-mortem* biopsy tissue did not show evidence for a great loss of these cells (Lowe et al., 1988), which suggests that the clinical features and pathological changes in AD brain are not dependent on the loss of GABA-ergic neurones.

In AD brain, $GABA_A$ receptor binding is decreased by a modest amount in frontal cortex when assessed by membrane binding, and also in posterior cingulate, when assessed by autoradiographic analysis (Lloyd et al., 1991; Vogt et al., 1991). Benzodiazepine binding in the hippocampus was slightly reduced in CA1, subiculum and entorhinal cortex (Jansen et al., 1990). In another study, binding to the $GABA_B$ receptor was reduced to 50-60 % in frontal cortex, although no changes were described for $GABA_A$ binding (Chu et al., 1987).

1.10.5 Excitatory amino acids

Many presumed markers of glutamatergic neurones are rather non-specific. Nevertheless, a number of studies have reported reduced glutamate concentrations in AD brain (Arai et al., 1985; Ellison et al., 1986; Sasaki et al., 1986). Moreover, the glutamate content of temporal lobe biopsy samples from AD patients was also reduced, and the value for individual subjects related to the density of pyramidal neurones in layer III of the neocortex (Lowe et al., 1990). Additionally, Hyman et al., (1987) reported an 80 % reduction in glutamate concentration in the terminal zone of the perforant pathway. Consistent with this, decreased glutamate staining was observed in the molecular layer of the dentate gyrus (Kowall & Beal, 1991), and the same study also reported reduced numbers of glutamate and glutaminase immunoreactivity pyramidal neurones in the CA fields of the hippocampus.

In a study using specimen obtained *ante-mortem* (by biopsy) approximately three years after emergence of symptoms, and promptly *post-mortem* some ten years after onset. Early in the disease a slight elevation in aspartic acid concentration of cerebral cortex was observed in

patients suffering from AD. A reduction in glutamate concentration of a similar magnitude was observed. Later in the disease evidence of glutamatergic neurone loss is provided by the finding that in many regions of the cerebral cortex the Na^+-dependent uptake of D-[^{3}H] aspartic acid was almost always lowest in AD subjects compared with control (Procter et al., 1988)..

Other studies have examined a number of putative markers of glutamatergic neurones such as high affinity [^{3}H] D-aspartate uptake in frozen material (Hardy et al., 1987) and Na -dependent [^{3}H] D-aspartate binding (Cowburn et al., 1988). K -stimulated Ca -dependent excitatory amino acid (EAA) release could not be demonstrated in brain tissue from promptly performed autopsies from AD patients or demented controls (Procter et al., 1988b). This was considered to reflect an epiphenomenon, perhaps related to terminal hypoxia. Immunocytochemical localisation of glutaminase immunoreactive neurones may be a more reliable method. Loss of glutaminase positive neurones has been noted in the cortex of AD brains (Akiyama et al., 1989; Kowall & Beal, 1991).

Evidence of deficits of postsynaptic EAA receptors is conflicting. Some investigators have observed that areas of control brains with the highest density of EAA receptors are those which undergo the most severe degeneration in AD (Young & Penney, 1994). The binding of [^{3}H] glutamate has often been used to assess EAA receptors. Greenamyre et al., (1985) reported reductions in neocortical areas of AD brain compared to control brains and brains from patients suffering from Huntington's disease, that appeared to be primarily attributable to loss of N-methyl-D-aspartate (NMDA) receptors. Similar deficits in NMDA receptors were reported in autoradiographic studies of hippocampal AD tissue, particularly in CA1 pyramidal cell and molecular layers (Greenamyre et al., 1987), although other investigators have reported more widespread loss of NMDA receptors in the hippocampus (Maragos et al., 1987). Autoradiographic studies have described abnormalities in NMDA receptors in AD (Young & Penney, 1994). Moreover, several studies have reported reduced sensitivity of the NMDA receptor to glycine in AD (Steele et al., 1989; Procter et al., 1989; Procter et al., 1991), which

may reflect differential loss of a glycine sensitive NMDA receptor subtype, or selective alteration of glycine site coupling to the receptor.

Studies of non-NMDA receptor subtypes are also conflicting. [^{3}H] kainate binding was significantly increased in deep layers of frontal cortex and correlated with plaque number in the same area (Chalmers et al., 1990), but no change in [^{3}H] α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) binding was found. However, decreases in both kainate (Geddes & Cotman, 1986; Penney et al., 1990) and AMPA (Penney et al., 1990) receptors were found in AD hippocampus, in agreement with Greenamyre et al., (1987), who used [^{3}H] quisqualate to label both kainate and AMPA receptors, but in contrast to *in-situ* hybridisation histochemistry studies for kainate/AMPA receptors, which described modest increases of receptor mRNA in CA4 and subiculum (Harrison et al., 1990). In caudate nucleus membranes prepared from brains of examples of AD and controls no difference in binding of [^{3}H] kainate was eminent (Pearce and Bowen, 1984) Also metabotropic receptors were found to be reduced (Dewar et al., 1991).

Discrepancies in the study of postsynaptic EAA receptor density in AD may be caused by several factors; differences in experimental technique (e.g. autoradiographic or homogenate binding study); severity of the disease or inadequate control for epiphenomena such as *post-mortem* delay, age match of AD and control tissue or agonal state. However, taken together, data indicate that it is not unlikely that a deficit in the glutamatergic system exists in AD brain.

1.11 Neurotransmitter control of APP-metabolism

One of the very exciting findings of recent years is that the processing of APP can be influenced by first messenger systems via stimulation of neurotransmitter receptors in cell lines (for review see: Nitsch et al., 1994)). The first study which focused on this possibility was done by Nitsch et al., 1992). These experiments in human embryonic kidney cells transfected with cDNA encoding for human muscarinic acetylcholine receptor (mAChR) showed that activation of both M_1 and M_3 AChR's with the muscarinic agonist carbachol significantly increases the release of soluble large N-terminal APP derivatives, lacking the C-terminus (APP_s). These initial findings were subsequently confirmed in a rat pheochromocytoma PC-12 cell line transfected with the muscarinic m_1 receptor gene, and extended by the observation that stimulation of interleukin 1 receptors also increase the release of N-terminal APP derivatives (Buxbaum et al., 1992). This is also a feature of glutamatergic metabotropic receptors (Lee et al., 1995). The general picture which emerges from these experiments is that receptors linked to the Phospholipase C (PLC) pathway can stimulate secretion. The PLC pathway results in the formation of diacylglycerol (DAG), which is a physiological activator of protein kinase C (PKC). DAG is formed by phospholipid hydrolysis following cell surface receptors that are coupled, via G protein, to PLC activation (for review see: Nishizuka, 1992) That secretion of APP is linked to activation of PKC is suggested by experiments using different PKC inhibitors. For example, calphostin C, chelerythine choride and staurosporine all inhibit neurotransmitter stimulated APP secretion. In addition, experiments with fibroblasts transfected with the cDNA encoding for the PKC_α isoenzyme clearly indicated that this PKC subtype can stimulate APP_s secretion, and phorbol-ester-mediated activation of PKC increased the secretion of APP_s from cells in culture (Slack et al., 1993; Caporaso et al., 1992; Buxbaum et al., 1990). Another interesting finding, and possibly with even greater implications for the treatment of AD, is that

stimulation of secretion of APP_s can be accompanied by a decrease in the formation of ßA4. The way in which this is achieved is that the enzyme which cleaves APP, making it "ready" for secretion, will split the protein in the middle of the ßA4 sequence, thereby precluding the formation of amyloid.

The regulation of biochemical pathways in the intact brain is, in many ways, much more complex than that in cell culture model systems. For example, biochemical reactions in brain cell *in situ* can be affected simultaneously by many neurotransmitters and neuromodulators. This is a situation very different from that created in culture cells transfected with one neurotransmitter receptor, where one first messenger system can be tested at the time. Some experiments have aimed at investigating the processes regulating APP secretion in an experimental setup which mimicks the *in vivo* situation. For example, Nitsch et al., (1993) showed that electrical stimulation of hippocampal slices resulted in a two-fold increased release of APP_s within 50 minutes, and that this release was related to the stimulation frequency used. Also tetrodotoxin blocked this depolarisation-induced increase. Although neurotransmission deficits are noted in other diseases which do not show accelerated formation of plaques (for example, Parkinson's Disease, Huntingdon's Disease and system atrophies) and because in AD plaques are formed in regions of the brain which are relatively spared from neuronal damage and which can be expected to show no neurotransmitter abnormalities (Nitsch et al., 1994). Although neurotransmitter deficiencies may not be the principal cause of the formation of plaques, they may be an aetiological factor in its formation. Also, an additional question which still awaits a conclusive answer is how exactly ßA4 contributes to the development of the histological hallmarks of the disease.

1.12 Risk factors

1.12.1 Age

Age seems to be the major risk factor of getting AD (see also section 1.5.1).

The risk of getting dementia increases exponentially.

One of the factors which may be responsible for this increased risk may be the increase in markers of oxidative stress, which may accelerate the formation of neurofibrillary tangles and senile plaques, and increase the vulnerability of neurones in general.

1.12.2 Genetic mutations: linkage to chromosome 21

Since the discovery that the gene encoding for APP is localised to chromosome 21, intensive research has identified a number of genetic mutations that are associated with the incidence of AD in a small percentage of sufferers. At least six missense mutations have been detected within or immediately next to the amyloid encoding region of the APP gene in families with autosomal dominant, early onset AD (Goate et al., 1991). Although most of the known missense mutations in exons 16 and 17 of APP are strongly linked to AD, the mechanisms by which they predispose for the disease remain unresolved. However, this evidence together with the observation that patients suffering from Down's syndrome, who have three copies of chromosome 21, invariably develop Alzheimer-like symptoms early in life, led some investigators to propose that mismetabolism of APP was directly linked to the disease.

1.12.3 Genetic mutations: linkage to chromosome 19

There are three common forms or alleles of the apolipoprotein-E gene, namely apo-E2,

apo-E3 and apo-E4. The role of apo-E4 in AD was first suggested when studies appeared showing apparent linkage of the development of AD with a site on chromosome 19 (Pericak-Vance et al., 1991). Strittmatter et al. (1993) were the first to report an allele frequency of apo-E4 of 50 % in 30 affected familial AD patients. A study of 95 affected late onset familial AD individuals revealed 80 % who had the E4 allele, with an apparent gene dosage effect (Corder et al., 1993). Since then, several studies have shown that in sporadic AD cases between 45 and 65 % of individuals carry at least one apo-E4 allele (as reviewed, Katzman, 1994). Between 25 and 40 % of all AD cases can be attributed to apo-E4, making this the most common risk factor for AD.

The mechanism by which apo-E4 allele predisposes to the development of AD is currently unclear. However, Strittmatter et al. (1993) have shown that apo-E4 avidly binds to soluble amyloid in an *in vitro* setting. If this also occurs *in vivo* too much apo E4 could accelerate the formation of plaques. This is in keeping with findings that brains of patients homozygous for the E4 allele contain increased amounts of vascular amyloid, and increased numbers of neuritic plaques compared to those homozygous for the E3 allele (Schmechel et al., 1993). Additionally, Strittmatter et al., (1994) focused attention on the fact that apo-E3 binds to tau protein, possibly slowing the initial rate of tau phosphorylation and self-assembly into PHF, while apo-E4 does not bind tau.

1.12.4 Genetic mutations: linkage to chromosome 14

A recent study suggests that a third gene bearing missense mutations in early-onset AD is located on chromosome 14. Results provide strong evidence that mutations in the S182 gene are the cause of early onset familial AD in some pedigrees, but both the physiological role of the gene and the reason why its mutations confer a dominantly inherited AD phenotype remain

unknown at this point in time. (Sherrington et al., 1995)

1.12.5 Genetic mutations: linkage to chromosome 1

Recently an additional candidate gene for the chromosome 1 AD locus was identified, called STM 2 (Levy-Lahad et al., 1995). The predicted amino acid sequence for STM 2 was very similar to that of chromosome 14 AD gene (S182), but the function of the gene product is at this point in time unknown.

1.13 Aims of the studies described in the thesis.

The main aim of the studies described in this thesis is to test the hypothesis that modulation of receptors located on cortical pyramidal neurone perikarya can modulate excitatory amino acid release from terminals of these neurones. Three categories of drugs were studied, an excitatory amino acid receptor agonist, an antagonist of the serotonin 1A receptor and cholinomimetics.

Hypotheses which were tested in this thesis are:

I: Topical application to the frontal cortex of an excitatory amino acid receptor agonist will increase concentrations of glutamate and aspartate in the striatum.

II: 5-HT_{1A} receptor antagonists block the action of an endogenous hyperpolarising agent and potentiate the effect of an excitatory amino acid receptor agonist.

III Cholinomimetics can increase the concentrations of glutamate and aspartate, and thereby the activity of cortical pyramidal neurones

IV: Increasing neuronal activity increases the secretion of amyloid precursor protein *in vivo*.

It is hoped that the studies performed, by deepening the insight in the pharmacological regulation of cortical pyramidal neurones, will help in the development of novel therapeutic approaches for the treatment of AD.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Dialysis

2.1.1 Development of microdialysis

(see for reviews: Benveniste & Huttemeier, 1990; Parsons & Justice, 1994).

In order to measure substances in the extracellular space of the brain, Gaddum, (1961) was the first to use a technique called "push pull", creating a moving film of fluid, continuously in contact with the extracellular space.

The first attempt to measure substances in the extracellular space by a technique which resembled more the microdialysis technique of today, was made by Bito et al., (1966), who implanted, what they called a "dialysis sac" containing saline into the subcutaneous tissue of the neck and the parenchyma of the cerebral hemispheres of dogs. After a period of ten weeks the sac was removed, and analysed for content of amino acids. These experiments were the first that used the idea of a "compartment" surrounded by a dialysis membrane which equilibrates to the extracellular environment. Delgado et al., (1972) developed a "dialytrode" which shows great similarity to the present microdialysis probes. The technique is widely used now, exemplified by an exponential increase in publications which have used microdialysis.

2.1.2 Features of microdialysis

In microdialysis a probe made of a semipermeable membrane, is used to mimic the passive function of a capillary blood vessel. The general aim is to enable substances in the extracellular space to diffuse into the probe, thereby giving an indication of their concentration, and change in concentration over time. The advent of high performance liquid chromatography (see section 2.6) has enabled scientists to measure minute amounts of substances of interest in the dialysate.

Microdialysis can sample the extracellular space in the brain, but additionally recent years has seen studies in almost every organ in the body. One major advantage of microdialysis over techniques such as the slice and synaptosomes is that it is performed in the intact animal, leaving the normal anatomical connections intact.

One important "side-effect" of microdialysis is caused by the fact that it is an invasive technique, and hence, tissue disturbance will occur. However, when implanted slowly into the tissue, microdialysis causes minimal damage to the blood-brain barrier (Benveniste et al., 1984; Tossman & Ungerstedt, 1986).

2.1.3 Comparisons to push-pull

Microdialysis is comparable to push pull in that both techniques attempt to sample the extracellular space. In essence, a microdialysis probe is a push-pull cannula with a dialysis membrane applied over its tip. This however makes a great difference in that, while in push pull it is always necessary to balance in and outflow, in microdialysis there is no such need. In a push pull experiment it is easy either to apply either too much "push", which will introduce too much fluid into the brain, or to "pull" too hard, which may cause damage to brain tissue. It follows therefore that microdialysis is likely to cause less damage to brain tissue surrounding the probe than the push- pull approach.

Another major difference between dialysis and push pull is that the barrier formed by the microdialysis membrane forms an effective barrier for molecules which are larger than the pores of the dialysis membrane used. The sampling of the extracellular fluid by microdialysis offers a selectivity not available with push-pull. Membranes with different pore sizes are available which opens up the possibility of excluding molecules of a certain size.

2.1.4 Principles of microdialysis

Microdialysis seems a very simple technique. A microdialysis probe is introduced into the tissue and perfused with a perfusion fluid which closely resembles in composition the extracellular fluid. This fluid is slowly moved through the probe to enable equilibration with the extracellular fluid to be established. Problems concerning the interpretation of microdialysis experiments stem from the complex interactions between the probe and the tissue it is positioned in.

2.1.4.1 Interaction between probe and tissue

By introducing a microdialysis probe, brain tissue will be traumatised. This initial lesion causes a rise in most substances measured. This high concentration declines to a lower, steady concentration within 30 minutes after implantation (Benveniste & Huttemeier, 1989). The reason why initial concentrations are high is not completely clear, but may relate to excessive release from cellular stores, due to rupture of cellular structures. After approximately 30 minutes a new steady state for most substances is established because of drainage through the probe (Lazarewicz et al., 1986; Amberg & Lindefors, 1989 ; Benveniste & Huttemeier, 1989). In general terms the degree of initial damage seems to be influenced by the speed with which the probe is introduced into the brain or any other organ.

Another consequence of introduction of a dialysis probe into brain tissue is that the tissue around the probe does not function normally. This is exemplified in an increase in glucose metabolism, decreased blood flow and disturbed neurotransmitter release after implantation of the dialysis probe (Benveniste et al., 1987; Drew et al., 1989; Osborne et al., 1990; Osborne et al., 1991).

Implantation of a dialysis probe is normally accompanied by a disturbance of the blood

brain barrier. However, this disturbance is normally short-lived, in general the blood brain barrier around the microdialysis probe is reestablished shortly (30 minutes to 2 hours) after implantation (Benveniste et al., 1984; Tossman & Ungerstedt, 1986). The amount of trauma which is associated with the introduction of the microdialysis probe probably determines the time it takes for the tissue around the probe to normalise.

2.1.4.2 Diffusion and recovery

The main reason why microdialysis works is that substances which are present in a higher concentration outside the probe than inside the probe will diffuse into it. A logarithmic concentration gradient is thus generated towards and away from the probe. The direction and the speed of the diffusion depends, amongst other factors, on the difference in concentration of a given compound between the extracellular space and that in the dialysis fluid. For example, if Ca^{2+} is left out from the perfusion fluid, the extracellular fluid around the probe is depleted of Ca^{2+}, reducing release of neurotransmitter release (Imperato & Di Chiara, 1984; Westerink & de Vries, 1988). It is therefore of extreme importance that the composition of the dialysis fluid resembles the extracellular fluid as closely as possible (Moghaddam & Bunney, 1989).

Recovery of substances from the extracellular space depends on many factors. Important parameters determining this are: length of dialysis membrane, composition of the perfusate, flow rate of the perfusate through the probe and physical properties of the substance of interest (Amberg & Lindefors, 1989; Benveniste & Huttemeier, 1989; Lindefors et al., 1989; Bungay et al., 1990). For small molecules the rate limiting factor of recovery is the speed of diffusion through the extracellular fluid, rather than diffusion through the membrane (Amberg & Lindefors, 1989).

Normally relative recovery (concentration recovery) and absolute recovery (mass recovery) are distinguished (Ungerstedt, 1984). Relative recovery is the concentration of a particular

substance in the perfusate expressed as a percentage of the concentration in the medium. Absolute recovery is the total amount of the substance recovered during a period of time. In the *in vivo* setting, relative recovery remains constant as long as the perfusion conditions remain the same. However the absolute recovery changes with the amount of substance being produced in the tissue.

2.1.5 The microdialysis probe

Three types of microdialysis probe have been used, the dialysis membrane on its own, the loop probe and the concentric probe. The dialysis membrane on its own is a convenient tool to investigate changes in the concentration of compounds in the extracellular space for example in the intestinal wall (Bunnett et al., 1984). It has also been used in studies in small peripheral organs like the adrenal gland (Jarry et al., 1985) or ovary (Jarry et al., 1990). To limit the area of dialysis, the tube may be partially covered by epoxy or an outer impenetrable tube (Ungerstedt & Pycock, 1974; Imperato & Di Chiara, 1984; Tossman et al., 1985). The advantage of using the dialysis tube *per se* is that it is simple to make, and once implanted it follows the movements of the organ. A drawback is that it is necessary to make holes for both entry and exit and that the implantation procedure causes unnecessary damage to skull, muscles and brain regions *en passant.*

The loop probe consists of two parallel metal tubes, connected by a dialysis tube forming a loop (Zetterström et al., 1983). In order to stretch the loop during inplantation into the tissue a stylus is placed inside or alongside one of the metal tubes reaching all the way to the distal end of the loop. In order to avoid that the bend of the loop kinks and blocks the passage of liquid a thin fibre can be placed inside the bend of the loop. The loop probe is easy to make and position in a tissue, however, the two parallel tubes increase its size, and thereby the damage of the tissue.

In the concentric probe, a piece of dialysis tubing is sealed by glue at one end forming the tip of the probe. The other end is usually glued into a steel tube which forms the shaft of the probe. A thin inner cannula extends through the shaft and dialysis tube all the way to the tip. The material may be metal, fused silica or plastic. The perfusing liquid enters the proximal end of the inner cannula and flows distally all the way to its end where it changes direction and returns in the space between the inner cannula and the membrane where the microdialysis takes place. The proximal end is designated in such a way that the inlet and the outlet are separated. The concentric probe is the most difficult to make. It can be made very thin and the length of the tip can be varied from mm to cm in length. It is the only probe suited for the introduction into the tissue through a guide cannula. A drawback is that air bubbles may easily be trapped inside the membrane, limiting the area of diffusion. The membrane used in dialysis experiments should be bio-compatible and as inert as possible in order not to interfere with the passage of molecules. However, this can always be tested in an *in vitro* experiment.

2.1.6 Using anaesthetised or awake animals.

Microdialysis can be performed acutely on anaesthetised animals or on awake animal with chronically implanted probes and guide cannulas. Anaesthetising the animal is normally a requirement if complicated experiments are planned, such as local injections, electrical stimulation or in experiments requiring dissections such as the exposure of the spinal cord, cerebral cortex or a peripheral nerve.

One of the great advantages of microdialysis is that experiments on awake animals are relatively easy. However, the advantage of not using an anaesthetic agent is balanced by the disadvantage of working on an animal susceptible to all kinds of influences ranging from the pain of the implantation and the restraint stress of tubing and wires, to reactions in response to a new environment. It is conceivable that behaviour *per se* induces secondary changes in

transmitter release.

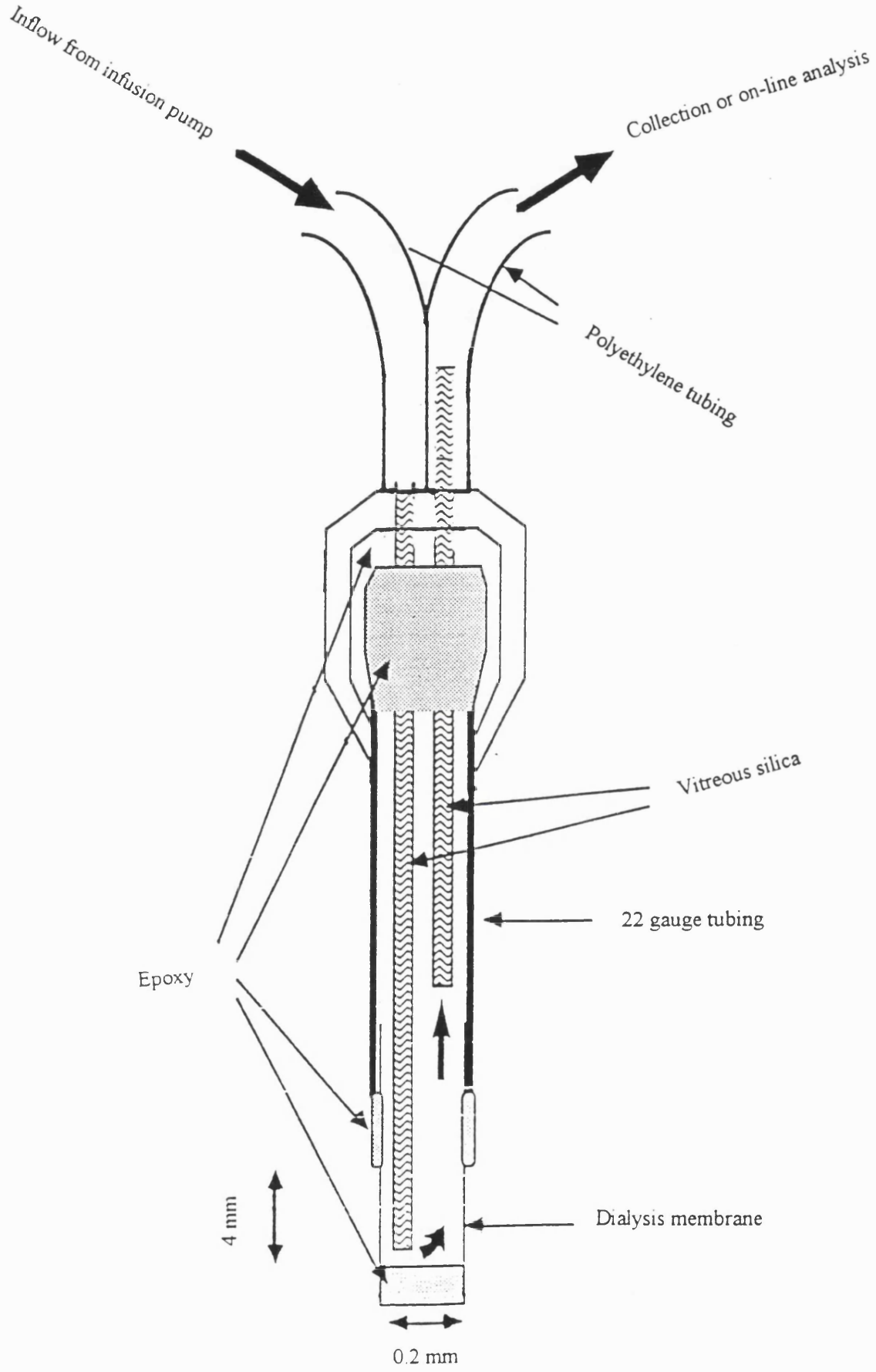
2.1.7 The microdialysis probe used in this thesis

A concentric probe (diameter 200 μm) was implanted in the striatum. The microdialysis probe was a modification of those previously described by Hutson et al., (1988) and was made as follows: On mm paper two rows of blue tack were stretched out. Metal tubing (22 G, 15 mm length, Cooper's Needleworks, Birmingham, W Midlands, UK) was placed on one row. Two pieces of fused silica tubing, length 2 and 3 cm respectively (0.19 mm outer diameter (OD). 0.075 mm inner diameter (ID); SGE Ltd, Milton Keynes, UK) were cut and the longest piece of fused silica inserted in the metal tubing so that 5 mm protrudes. The other piece is then inserted until 10 mm protrudes. The pieces of fused silica are glued together with epoxy resin (Araldite) at the point where they enter the metal tube, and left overnight to dry. The following day two pieces of polythene tubing (ID 0.28 mm; OD 0.61 mm 800/100/100/100, Portex Ltd, Hythe, Kent, UK) are cut with a sharp knife (6 and 3 cm respectively). The longer piece is gently manoeuvred over the shorter piece of fused silica protruding from the metal tubing and fixed on the second row of blue tack. The shorter piece of tubing is pushed over the longer piece of fused silica. The pieces of polythene tubing are then glued to the end of the metal tubing and left to dry overnight. Subsequently the remaining piece of fused silica is cut to 4 mm. A dialysis membrane (copolymer of acrylonitrile and sodium methallyl sulphonate, AN 69, 0.31 mm OD, 0.22 mm ID; Hospal Medical, New Brunswick, NY, USA) is pushed over the fused silica and inside the metal tubing. The membrane is cut, the tip is glued tight and the membrane is glued to the metal tubing. This final version of the probe was left to dry for 24 hours before use. Before the start of the experiment probes were connected to the perfusion pump and tested for leaks.

The perfusion fluid for dialysis was phosphate buffered saline (PBS tablets, Sigma, Poole, UK)

containing in mM: 137 NaCl, 1.3 $CaCl_2$, 1.3 $MgCl_2$, 5 KCl , pH=7.3, to give a modified PBS, (mPBS). In all cases the perfusion rate was 1 μl/min. The perfusion pump used was a Carnegie Medicin micro-infusion pump (Carnegie Medicin, Stockholm, Sweden) and a 1 ml syringe (Carnegie Medicin, Stockholm, Sweden). When changing of the perfusion fluid was required a liquid switch (Carnegie Medicin, Stockholm, Sweden) was used. In some experiments a Ca^{2+}-free medium was used to investigate the Ca^{2+}- dependency of the amino acid overflows observed. The dialysis fluid in these experiments contained (in mM) 138.9 NaCl, 1.3 $MgCl_2$, 5 KCl , pH=7.3 All drugs were dissolved in mPBS unless stated otherwise.

see next page for a schematic diagram of the dialysis probe used in this thesis
(Fig 2.1)


Inflow from infusion pump
Collection or on-line analysis
Polyethylene tubing
Vitreous silica
22 gauge tubing
Epoxy
Dialysis membrane
4 mm
0.2 mm

2.2 Push-pull

2.2.1 Push-pull perfusion

The first to describe the push-pull perfusion technique was Gaddum, (1961), who constructed a cannula consisting of two metal tubes which were stereotactically implanted in the brain. Fluid is pumped through the inner tube and removed through the outer tube. The result is that the perfusion fluid is in direct, and constant contact with brain tissue surrounding the tip of the cannula. Substances present in the extracellular space are taken up in the perfusion fluid, and can then be analysed. In the past, push-pull perfusion has been used to measure the release of a variety of compounds such as catecholamines, related metabolites, amino acids, peptides and acetylcholine from various brain areas (Cheramy et al., 1977; Nieoullon et al., 1977; Michelot et al., 1979; Vogt, 1975). Most often the experiments are performed in anaesthetised animals, although studies using conscious animals have appeared (Phillipu, 1984).

2.3 High Performance Liquid Chromatography.

Chromatography is an analytical technique which allows the separation and detection of minute quantities of matter in small samples. It is based on difference in solubility of the compound of interest between the mobile and the stationary phase. The mobile phase is forced through the fine particles of the stationary phase with pressure (hence high performance). The stationary phase is often silica particles onto which anionic or cationic groups (ion-exchange) or carbon chains which provide a non-polar surface (reverse phase) have been chemically bonded (Lindroth and Mopper, 1979).

Phosphate buffer used in this thesis was prepared as follows: 70.2 g of sodium dihydrogen orthophosphate ($Na_2PO_4.2H_2O$) and 17.9 g of disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$) were dissolved in 5 l distilled, deionised water and the pH adjusted to 5.7 or 4.5 with 0.1 M phosphoric acid. An equal volume of distilled, deionised water was added to give a stock solution of 0.05 M buffer, which was stored in Winchester bottles.

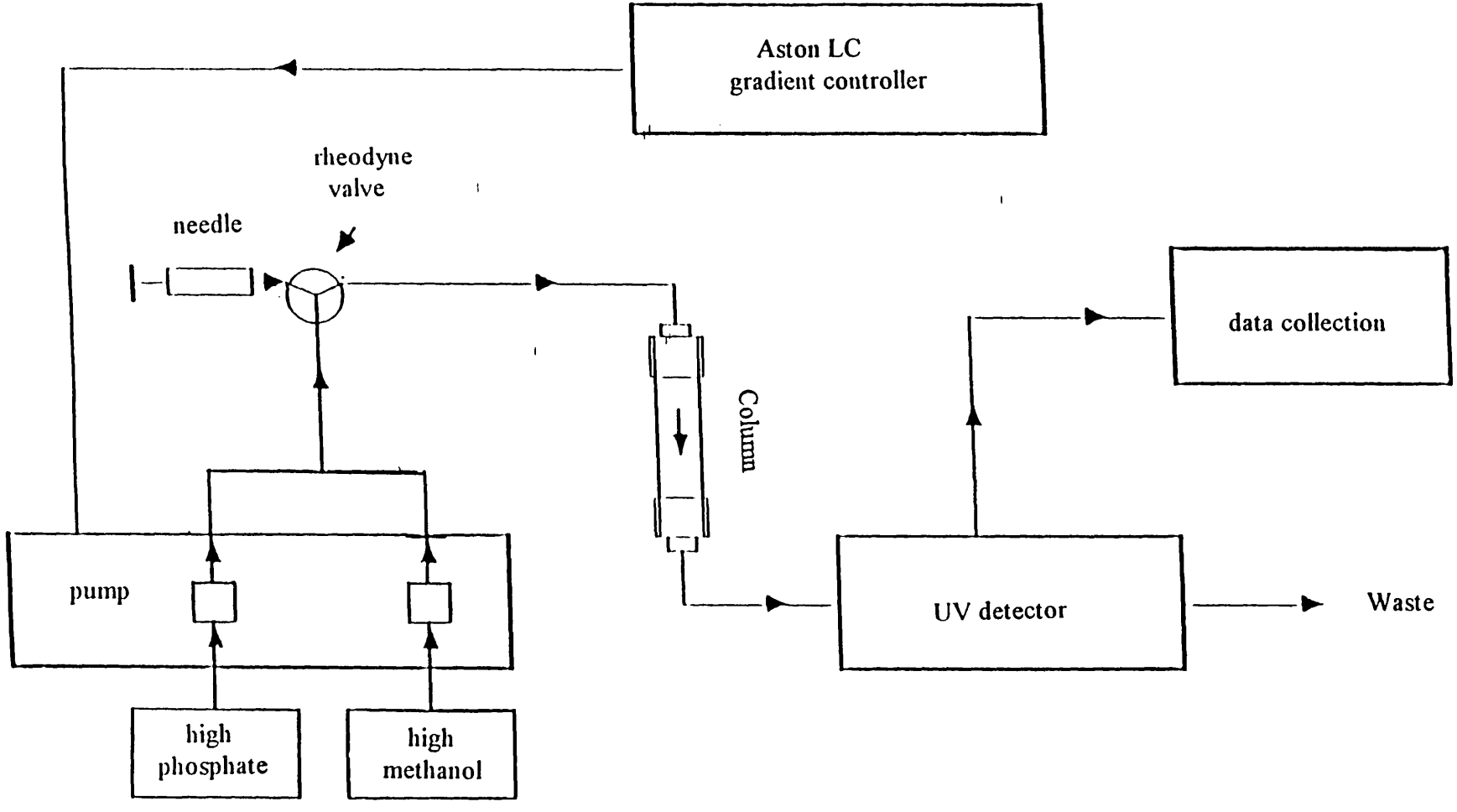
The eluent was a mixture of the phosphate buffer and methanol. For gradient elution, the mobile phase consisted of a low and a high methanol buffer. Buffer A consisted of phosphate buffer pH = 5.7, methanol, 80:20 (300 and 75 ml respectively), and buffer B consisted of phosphate buffer pH = 4.5, methanol, 20:80 (75 and 300 ml respectively). The mobile phases were filtered using a filter pump assembly (Millipore, UK, Ltd, Harrow, Middlesex, poresize 0.2 μm) and gassed for 5 minutes with helium before running it through the column. Fresh buffer was made up every day.

The HPLC apparatus consisted of a solvent delivery system (2 LKB Bromma 2150 HPLC pumps), a gradient controller (Aston LC, Aston Scientific, Aston Clinton, U.K.), injection valve with 20 μl loop (Model 7125 Rheodyne Inc, Cotati, CA, USA), a reverse phase octadecylsilane column of particle size 3 μm, 10 cm by 4.6 mm (Ultratechsphere 3 ODS, HPLC Technology, Macclesfield, Cheshire, UK.) together with a fluorometric detector (LDC Fluoromonitor III, LDC, Milton Roy, Stone, Staffordshire, UK, excitation 370 nm filter, emission 418-700 nm wavelength band pass). The flow rate was 1 ml/min. Peaks were identified by comparison of the retention times in authentic standard, and quantified by measuring peak areas.

To prepare amino acid standards or microdialysis samples for injection onto the column, o-phthaldialdehyde 50 μl was added to either 10 μl of a standard mixture or 10 μl of dialysate

sample, and thoroughly mixed. After a two minute reaction period the mixture was applied to the column.

See next page for a schematic diagram of the HPLC equipment (Fig. 2,2)


Aston LC
gradient controller
rheodyne
valve
needle
data collection
Column
pump
UV detector
Waste
high
phosphate
high
methanol
MOBILE PHASE

A gradient was used to elute amino acids. The duration and degree of change of buffer B is given below. A slope of one denotes a linear rise over a given period of time. A positive slope begins with an initial slow rise, which is steep at the end of the segment.

Initial % B = 5 %

Segment no.	time (min)	% B	Segment profile	Flow rate (ml/min)
1	2	5	1	1
2	2	10	1	1
3	2	15	1	1
4	6	47	1	1
5	6	52	1	1
6	2	85	1	1
7	3	5	1	1

TOTAL: 23 minutes

The level of detection of both ASP and GLU in striatal dialysate was between 0.1-0.4 pmol/10 μl. This was below typical baseline concentrations of ASP (1 pmol/10 μl) and GLU (2.5 pmol/10 μl). Figures 2,3 and 2,4 show standard curves for GLU and ASP, and figures 2,5 and 2,6 show typical chromatograms of a standard and a dialysis sample.

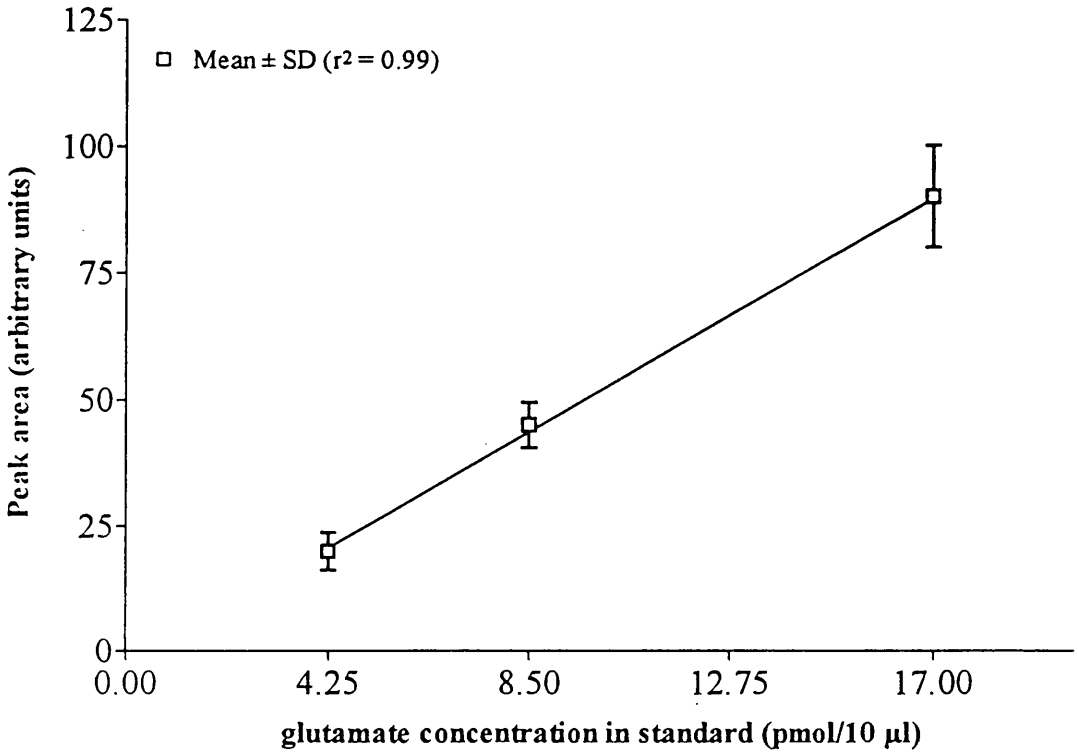


Fig. 2,4 Standard curve for glutamate

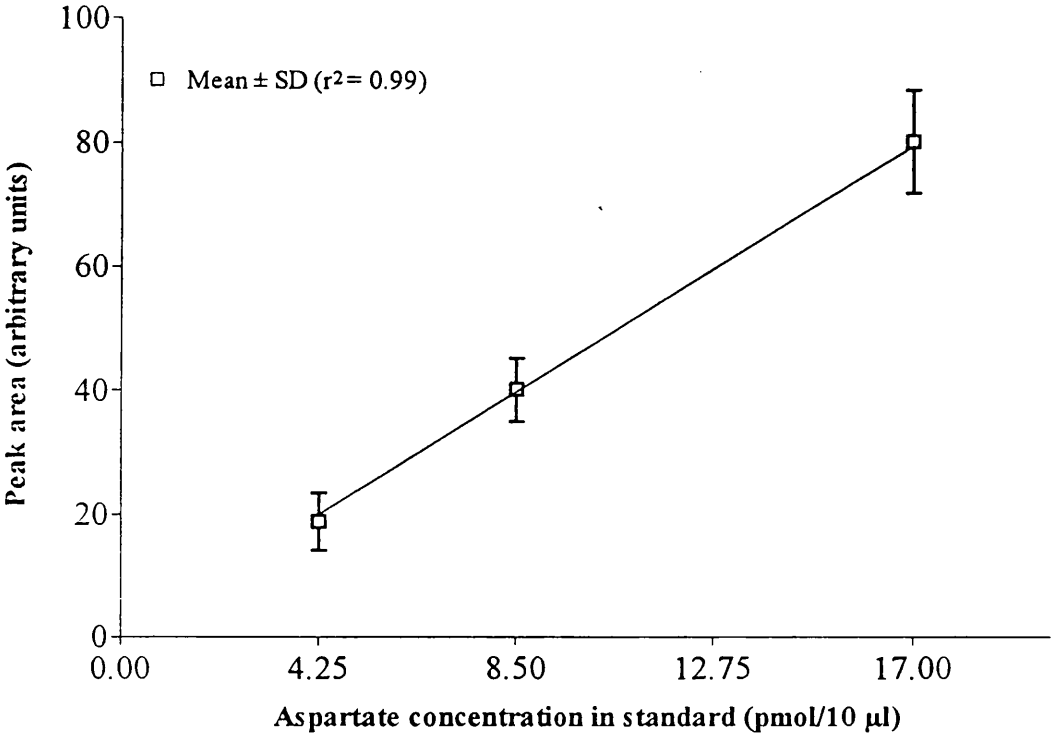


Fig. 2,5 Standard curve for aspartate

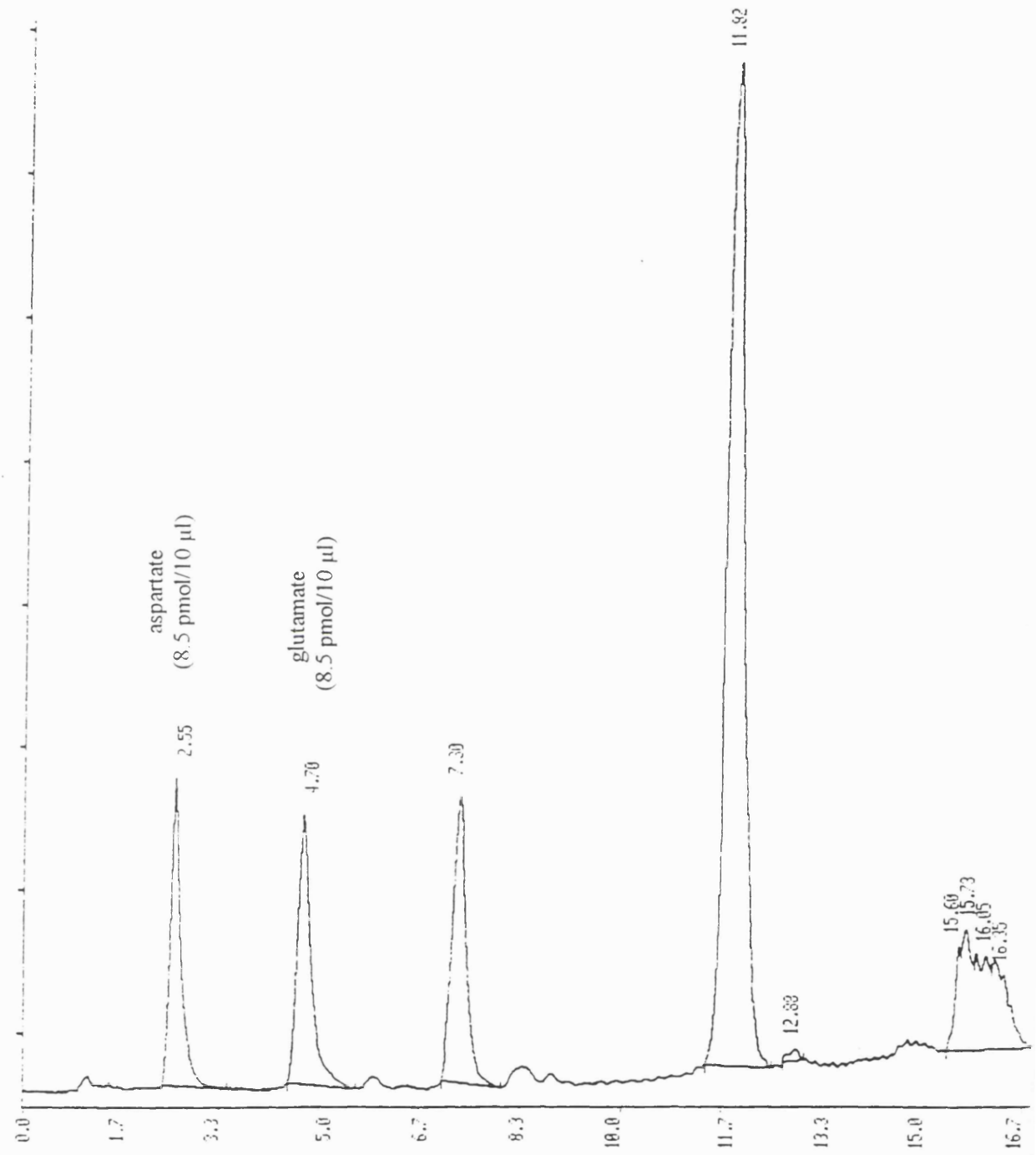


Fig. 2,6 Example of chromatogram of a standard

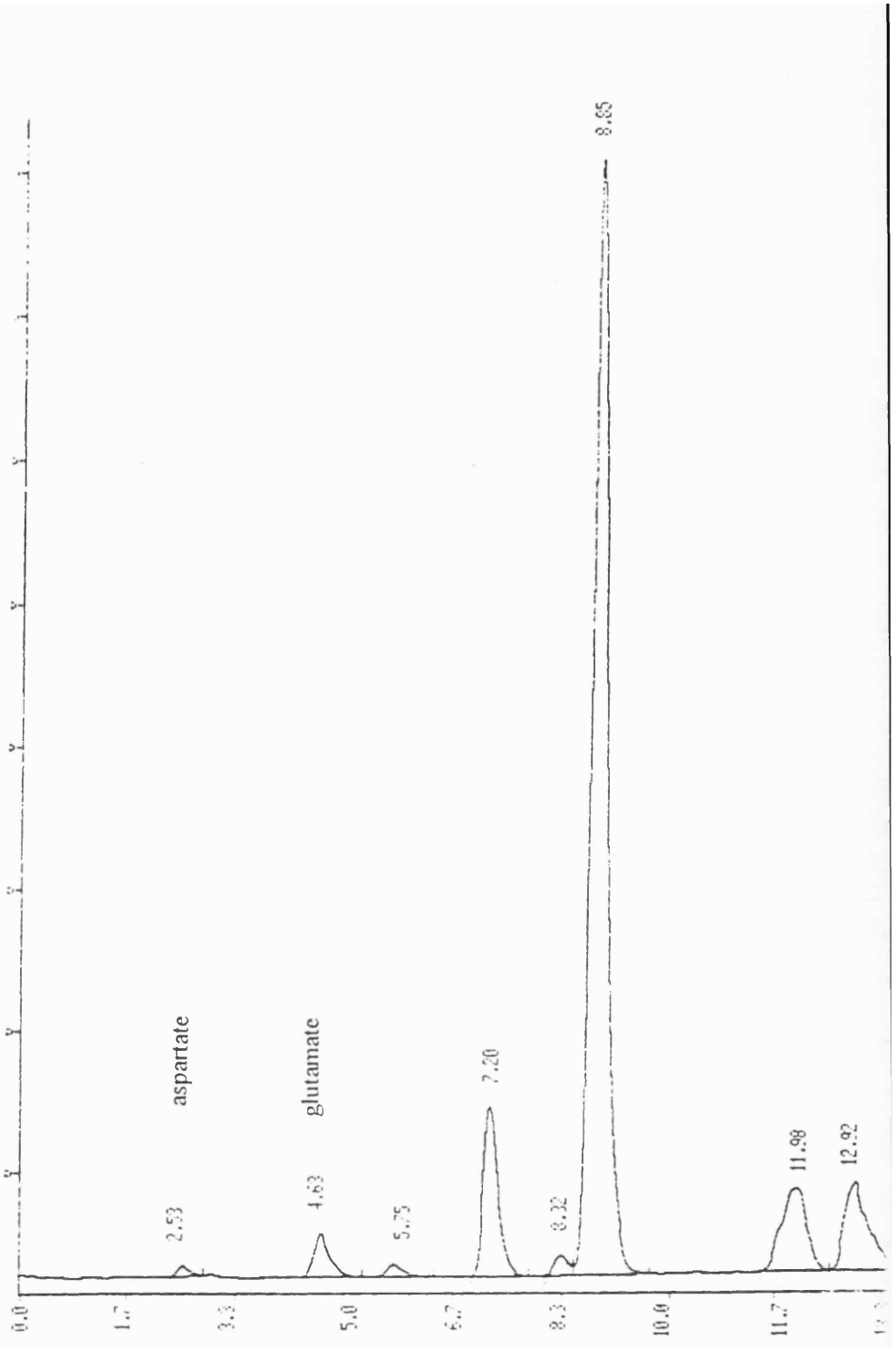


Fig. 2,7 Example of chromatogram of a microdialysis sample

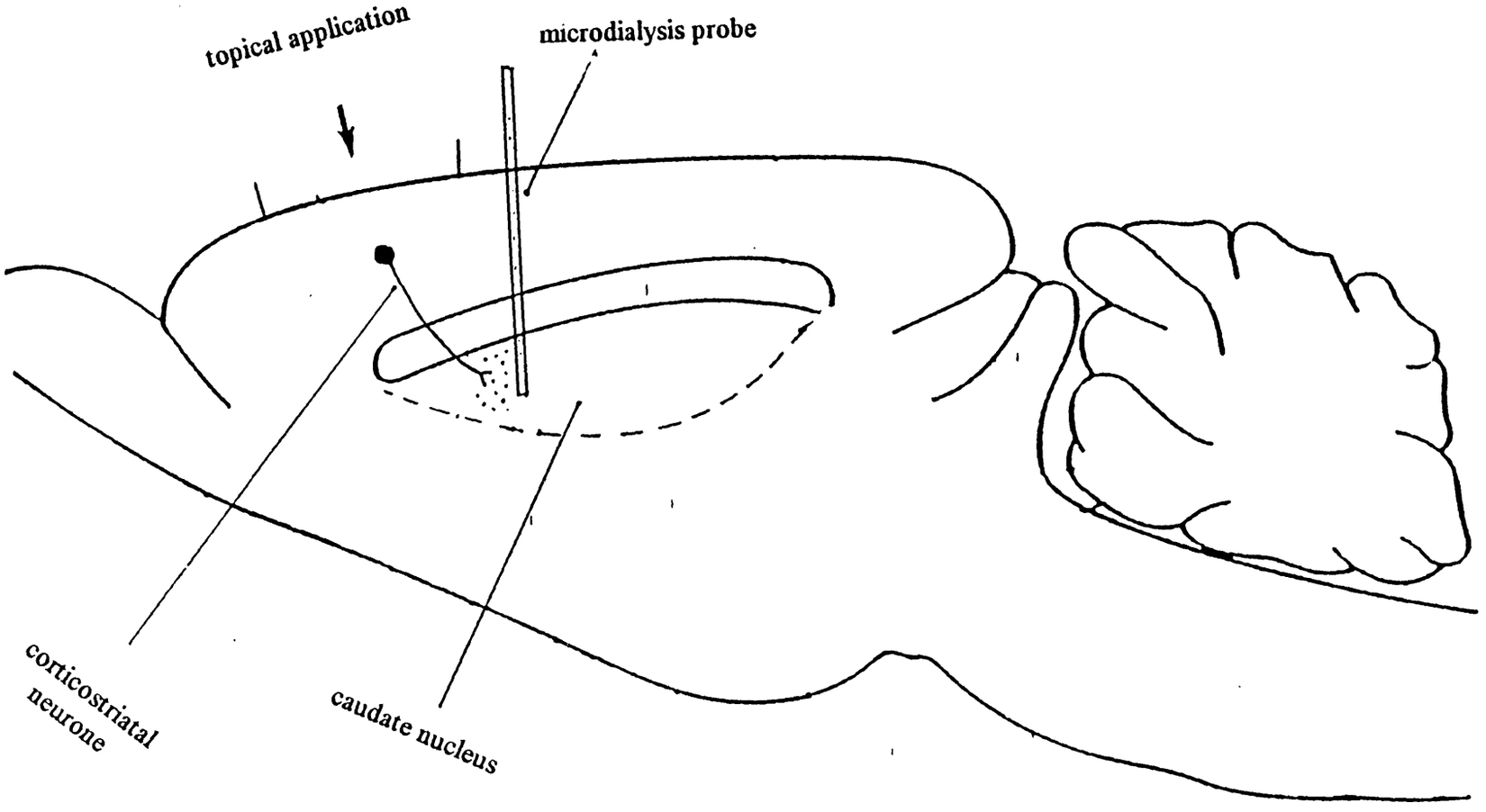
2.4 Animals

Male Sprague Dawley rats (Charles River, U.K) weighing between 200 and 250 grams were housed individually and had free access to food and water with a twelve hour light/dark cycle (lights on 0700h). Humidity and temperature were maintained at 45-50% and 20-21 °C, respectively. Before the experiments animals were allowed a one week period of adaptation.

2.5 Surgery

At the start of the experiment anaesthesia was induced with halothane (0.5 and 1.5 %) in a mixture of oxygen (flowrate 0.8 $ml.min^{-1}$) and nitrous oxide (flowrate 0.8 ml min^{-1}), until no reaction to tail and hind paw pinch was detectable. Under full anaesthesia animals were mounted in a Kopf stereotactic frame (incisor bar at - 3.3 mm). The skin was opened with a pair of scissors and the membranes overlying the skull were carefully removed. Part of the skull overlying the frontal cortex was carefully drilled away. The dura was removed with a small needle, with the aid of a magnifying glass. The resulting craniotomy typically filled with clear fluid. The craniotomy was protected from light with a tissue. A second burr hole was made over the striatum (coordinates with bregma as reference: AP 0 mm, L 2.5 mm, DV 7 mm from skull) to allow implantation of the microdialysis probe, which was slowly (over approximately one minute) lowered into the brain tissue. During the experiment the core temperature of the rat was kept at 37.7 °C with a heating pad linked to a feedback warm up system (Carnegie 150 temperature controller, Carnegie Medicin, Stockholm, Sweden).

See next page for schematic drawing of the experimental setup used in this thesis (Fig 2,7)


topical application
microdialysis probe
corticostriatal neurone
caudate nucleus

2.7 Determination of in vitro recovery of dialysis probe

To determine the *in vitro* recovery of the dialysis probes used in this thesis a solution (40 ml) was made of a mixed standard containing glutamate and aspartate (50 μM each; Sigma) in a glass container. This container was placed in a waterbath which was preheated to a constant 37 °C. Temperature of the fluid in the glass container was checked and when the temperature reached 37 °C the experiment was started. Probes were placed in the fluid and 10 minute samples were collected (flow rate 1 μl/min, same pump was used in these experiments as *in vivo*), without stirring. Samples were analysed for glutamate and aspartate concentrations using High Performance Liquid Chromatography (HPLC) (see section 2.6). This was compared to the signal given by the 50 μM mixed standard. The ratio of the two signals was calculated to give the relative recovery of the probes.

2.8 Treatment and sampling procedure

Four different routes of administering drugs were used in this thesis.

I) Topical (co)-application of drugs to the frontal cortex.

II) intramuscular injections.

III) intramuscular injections in combination with topical application.

IV) Application of drugs through the dialysis probe.

After implantation of the microdialysis probe the tissue was allowed a stabilisation period of 1 hour. During this period the implanted probe was perfused with mPBS at 1 μl/min. After this collection of samples was started. Dialysis fluid was collected into small vials and stored on ice.

Collection time per sample was 10 minutes yielding a total volume of 10 μl. A typical experiment consists of the collection of six baseline samples. At the beginning of each collection period the craniotomy over the frontal cortex is filled with 10 μl of vehicle. (for topical application experiments a 10 μl Hamilton syringe, Bonaduz AG, Switzerland, was used). At the beginning of the seventh sample drug dissolved in vehicle was administered. Eight more samples were collected (in total 15 samples per experiment were collected). At the beginning of sample eight to fifteen the craniotomy over the frontal cortex was filled with vehicle (mPBS). For experiments with peripheral injections, drug was dissolved in mPBS and injected at the beginning of the second collection period. In control experiments a equivalent volume of vehicle was injected.

2.9 Push-pull

A push-pull probe was attached to a Watson-Marlow pump (Falmouth, Cornwall, UK). In- and outflow were calibrated prior to starting the experiment. Typical flowrates were between 8 and 10 μl/min. After implantation the probe was perfused with a Ringer solution (containing in mM: NaCl 141; $MgCl_2$ 1.3; $CaCl_2$ 1.3; KCl 5). Collection time per sample was 30 minutes. After a stabilisation period of three samples (1.5 hours), tissue around the probe was perfused with Ringer containing 100 mM K^+ for five more samples (the perfusate in these experiments contained in mM: NaCl 46; $MgCl_2$ 1.3; $CaCl_2$ 1.3; KCl 100; n=6). In 4 control experiments normal Ringer was used for the whole experiment. Push-pull samples were collected in Amicon Centricon-30 tubes. Samples were stored on ice, 50 μl was taken out of the sample, deproteinised with acidified methanol and stored in - 70 °C until analysed for amino acid content (see section 2.6). The remainder of the sample was concentrated to a final volume of 50 μl. Samples were analysed for APP-like immunoreactivity using SDS-PAGE according to Webster

et al., (1994), and the APP amino terminal antibody 22C11 (epitope mapped to amino acids 60-100; Boehringer Mannheim, Lewis, UK). To the sample was added: 2.5 μl glycerol; 2 μl phenylmethylsulphonylglucoride 2 mM; 2.5 μl dithiothreitol 1 mM; 2.5 μl bromophenol blue 0.1% (w/v). 2.5 μl sample buffer {50 mM TRIS-HCl pH=6.8; 100 mM DTT; 4 % sodium dodecyl sulphate; 10 % glycerol; 10 mM EGTA}. This mixture was boiled for 4 minutes and 50 μl loaded on 10 % acrylamide gels at 180 Volts, then transferred on to a nitrocellulose membrane for immunodetection. Membranes were incubated with antibody in a dilution of 1:250. Blocking of non-specific binding sites was performed with phosphate buffered saline containing 4 % (w/v) milk powder. Membranes were washed three times for ten minutes. and incubated with the second antibody, peroxidase-conjugated-goat anti mouse IgG, 1:100 (1 μg/ml) (1.5 hours). Six second antibody washes were performed. Immunoreactive proteins were visualised (ECL kit; Amersham International) and scanned with a densitometer (EDC densitometer; Helena labs). The integral of total band density for each sample was divided by that of background. These values were then meaned.

2.10 Drugs and chemicals

The following drugs and chemicals were used in this thesis:

1) Bromophenol blue (BDH)
2) Calcium chloride (BDH)
3) Di-sodium hydrogen orthophosphate (BDH)
4) Di-sodium tetraborate (Borax) (BDH, Poole, England)
5) Dithiothreitol (BDH)
6) EGTA (Sigma)
7) Glycerol (Sigma)
8) Magnesium chloride (BDH)
9) Mercapto-ethanol (BDH)
10) Methanol (Analar, BDH)
11) Milk powder (Marvel dried skimmed milk)
12) N-Methyl-D-Aspartic acid (NMDA) (Sigma)
13) O-phthaldialdehyde (Sigma)
14) PD 142505-0028, (1-Azabicyclo [2.2.1] heptan-3-one, O-[3-(3-methoxyphenyl)-2-propynyl]oxime, (Z)-(=+/)-, ethanedioate (1:1) (salt) (kindly provided by Parke Davis)
15) Phenylmethylsulphonylglucoride
16) Physostigmine (Sigma)
17) Potassium chloride (Sigma)
18) Sodium chloride (BDH)
19) Sodium di-hydrogen orthophosphate (BDH)
20) Sodium dodecyl sulphate (Sigma)
21) Telenzepine dihydrochoride (RBI)

22) Tetrodotoxin (Sigma)

23) TRIS-HCL (Boehringer)

24) WAY 100135 (N-tert-butyl 3-4(2-methoxyphenyl) piperazin 1-yl-2-phenylpropanamidedihydrochloride) (Kindly provided by Wyeth)

25) 22C11 (epitope mapped to amino acids 60-100; Boehringer Mannheim)

2.11 Presentation of data and statistical analysis

Each sample was analysed for concentration of the two amino acids aspartate and glutamate and expressed in pmol in 10 μl perfusion fluid. In all Result Chapters except Chapter 6, where data were expressed as a percentage rise of baseline, statistical analysis was performed on the raw data. In Chapter 6 the percentage rise of baseline was calculated as follows. The mean of the first six samples was calculated and the value of each further sample was expressed as a percentage of this value. The values of all the animals in one group were then expressed as mean $\pm$ Standard Deviation.

If data were normally distributed parametric statistics were performed (repeated Measures ANOVA, comparison of individual means with one way ANOVA/LSD or T-test). If not normally distributed Kruskal-Wallis ANOVA and the Mann-Whitney-U test were used.

Using a different approach areas under the curve were calculated by adding the values for those samples where an increase in either ASP or GLU was observed. These values were then added to give a Mean $\pm$ SD. In Chapter 3 and 4 the values of sample 10,11 and 12 were added while in chapter 5 samples 2 until 15 were added, except in the case of the PD compound, where sample 9,10 and 11 were added. In Chapter 6 the values of samples 7,8,9,10,11 and 12 were added.

Statistical analysis was performed using ANOVA followed by either an LSD or a Mann Whitney U test as appropriate.

Chapter 3

NMDA, topically applied to the frontal cortex, induces an increase in extracellular concentration of glutamate and aspartate in the striatum.

3.1 Introduction

In the rat the corticostriatal projection system is organised topographically, such that practically all parts of the cortex project to immediate adjacent parts of the caudate-putamen. The existence of this system, the corticostriatal pathway, was first demonstrated in the rat (Webster, 1961) (see also section 1.8.2). It is possible to stimulate the corticostriatal pathway by topical application of drugs to the frontal cortex (Palmer et al., 1989). As a parameter of activity of this pathway, the release of glutamate and aspartate in the terminal field is monitored using a microdialysis probe. Microdialysis is a technique which allows the measurement of small molecular weight substances, present in the extracellular space of brain tissue and has been used in the past to study the release of neurotransmitters (see also section 1.12)

The purpose of this first series of experiments was to confirm previous findings that I) NMDA, a potent depolarising agent, when topically applied to the frontal cortex of the anaesthetised rat increases aspartate concentrations in the striatum; ii) to investigate whether a slight modification of the method would allow detection of NMDA-induced glutamate release, without coapplication of the GABA-antagonists bicuculline, picrotoxin and penicillin G and iii) investigate the Ca^{2+}-dependency and tetrodotoxin sensitivity of any amino acid outflow detected.

3.2 Materials and Methods

(The microdialysis paradigm, animals, surgery procedure and materials used are described in detail in the Materials and Methods section 2.1 -2.9)

The following drugs (in a volume of 10 μl) were applied to the frontal cortex, at the beginning of the seventh collection period:

A) vehicle (mPBS) n=8

B) NMDA (2 mM, n=7; 20 mM, n=6)

C) NMDA (20 mM), coapplied with tetrodotoxin 10 μM (n=5)

D) NMDA (20 mM), dialysis with Ca^{2+}-free medium (n=8)

E) 100 mM potassium (n=4)

3.3 Statistics

Data were expressed as pmol amino acid release in 10 μl microdialysate. Because the data were not normally distributed group means were compared using Kruskal-Wallis ANOVA and the Mann- Whitney-U test. Significance was taken at P values < 0.05.

3.4 Results

3.4.1 in vitro recovery of dialysis probes

In vitro recovery of dialysis probes (n = 5) was 32 $\pm$ 5.6 % (Mean $\pm$ SD) for glutamate and 35 $\pm$ 7.5 % for aspartate (see section 2.4).

3.4.2 Glutamate

NMDA (20 mM), topically applied at the beginning of the seventh collection period, increased significantly the concentration in the striatal dialysate of glutamate in sample 11, as compared to vehicle (vehicle = 2.6 $\pm$ 1.3 pmol/10 μl versus NMDA = 12.0 $\pm$ 6.0 pmol/10 μl, $P < 0.01$). This was also a feature when NMDA was applied in a concentration of 2 mM which increased glutamate in sample 11 (from 2.6 $\pm$ 1.3 pmol/10 μl to 7.8 $\pm$ 2.3 pmol/10 μl,

$P < 0.01$). A significant increase was also observed in sample12 (increase from 2.6 ± 1.5 pmol/10 µl to 4.8 ± 2.6 pmol/10 µl, $P < 0.05$; see Fig. 3,1). Tetrodotoxin reduced the NMDA (20 mM) in sample 11 significantly (from 12.0 ± 1.3 pmol/10 µl to 3.9 ± 4 pmol/10 µl, $P < 0.05$, Fig. 3,3).

Ca^{2+}-free medium perfused through the probe (perfusion was started immediately after implantation of the microdialysis probe) reduced the NMDA 20 mM effect in sample 11 (to 6.3 ± 3.9 pmol/10 µl , $P < 0.05$, Fig. 3,5).

100 mM potassium did not significantly increase glutamate concentrations (see Fig. 3,7).

3.4.3 Aspartate

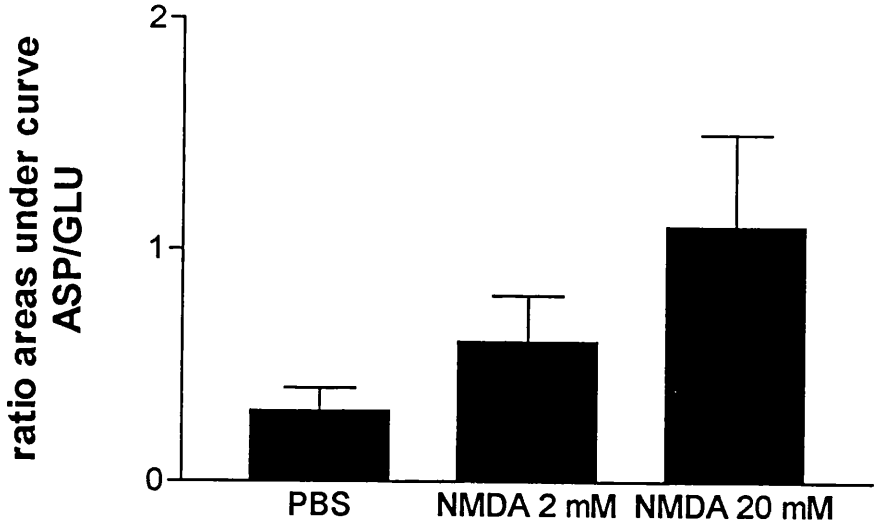
NMDA (20 mM), topically applied at the beginning of the seventh collection period, significantly increased dialysate aspartate concentrations in sample 9 (from 1.0 ± 0.8 pmol/10 µl to 3.8 ± 3.8 pmol/10 µl, $P < 0.05$), 10 (from 0.9 ± 0.7 pmol/10 µl to 3.1 ± 1.9 pmol/10 µl, $P < 0.01$), 11(from 0.8 ± 0.1 pmol/10 µl to 11.4 ± 2.5 pmol/10 µl, $P < 0.01$) and 12 (from 1.2 ± 0.6 pmol/10 µl to 2.6 ± 1.0 pmol/10 µl, $P < 0.05$). This was also a feature of NMDA (2mM), which increased aspartate in sample 10 (from 0.9 ± 0.7 pmol/10 µl to 2.9 ± 1.9 pmol/10 µl, $P < 0.05$), 11 (from 0.8 ± 0.1 to 6.2 ± 1.0 pmol/10 µl, $P < 0.01$) and 12 (from 1.2 ± 0.6 pmol/10 µl to 2.8 ± 0.8 pmol/10 µl, $P < 0.01$, see Fig. 3,2). Coapplication of tetrodotoxin with NMDA (20 mM) reduced the aspartate overflow in sample 11 (from 11.4 ± 2.5 pmol/10 µl to 3.1 ± 1.5 pmol/10 µl, $P < 0.05$, see Fig 3,4).

Omitting Ca^{2+} from the dialysis fluid resulted in a significant reduction of NMDA 20 mM effect in sample 11 (3.2 ± 1.0 pmol/10 µl, $P = 0.01$, Fig. 3,6). 100 mM potassium failed to increase significantly aspartate (see Fig. 3,8).

3.4.4 Aspartate and Glutamate area under the curve and ratio ASP/GLU

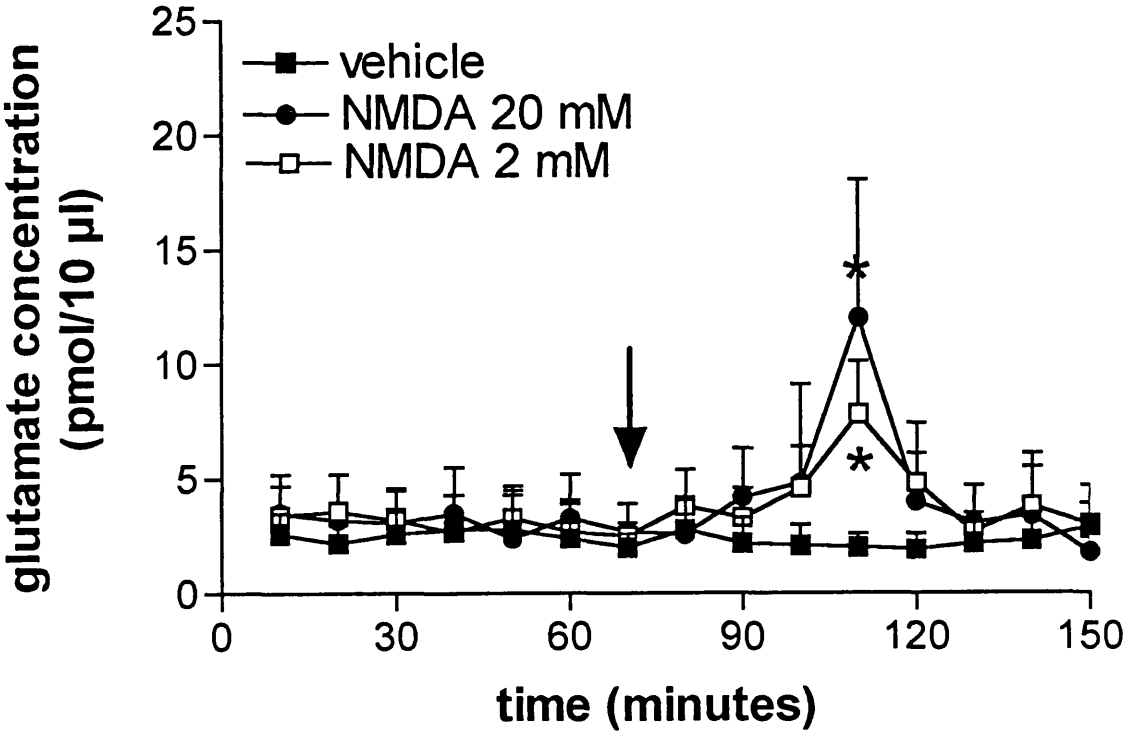
Treatment	area under curve ASP	area under curve GLU	ratio ASP/GLU
vehicle	2.7	9.0	0.3
NMDA 2mM	11.9	19.3	0.6
NMDA 20 mM	25.2	22.8	1.1
NMDA + TTX	8.2	9.5	0.9
NMDA +Ca2+free	5.0	10.5	0.5
High K^+	5.0	10.4	0.5

Fig 3.0



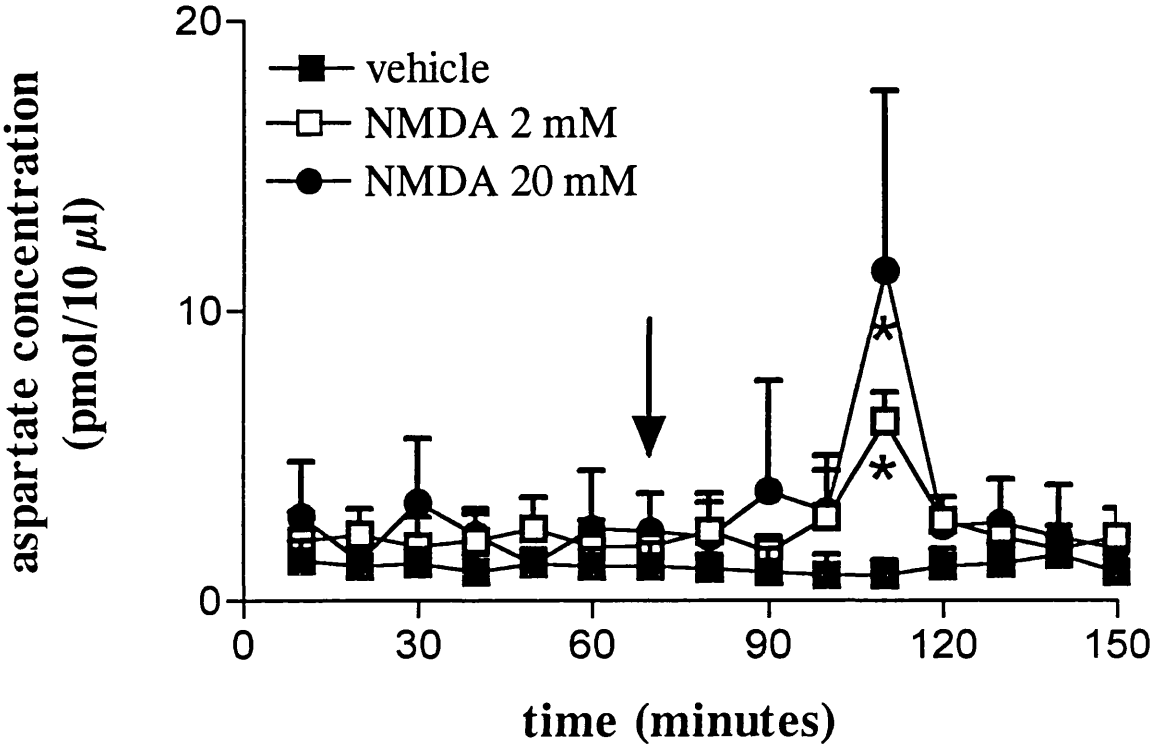
Relationship between concentration of NMDA, topically applied to the frontal cortex and ratio of ASP over GLU released in the striatum as a result. Linear regression analysis revealed a r value of 0.91, suggesting a linear relationship.

Fig 3,1



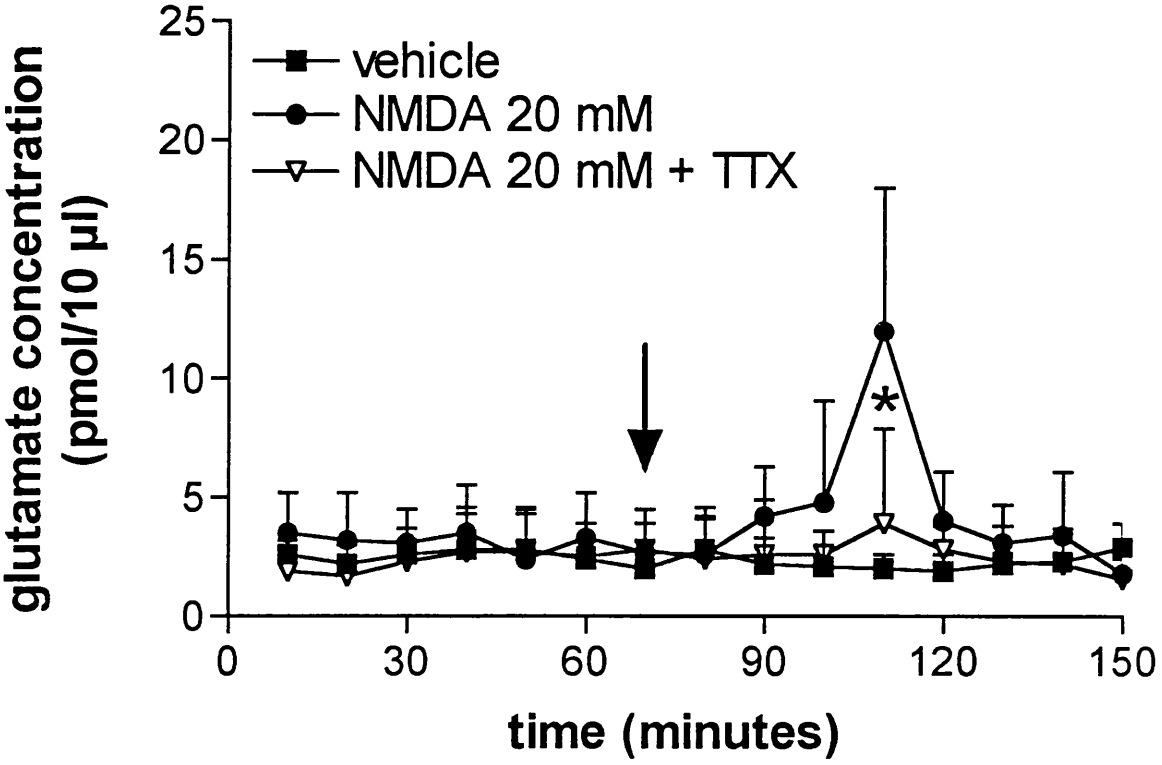
Effect of topical application of vehicle (arrow; n =7) or NMDA (arrow; 2 mM; n = 7; and 20 mM; n = 6) to the frontal cortex, on glutamate concentration in striatal dialysate. * = $P < 0.05$; ** = $P < 0.01$ significantly different from vehicle (Kruskal-Wallis ANOVA followed by Mann Whitney U-test).

Fig 3,2



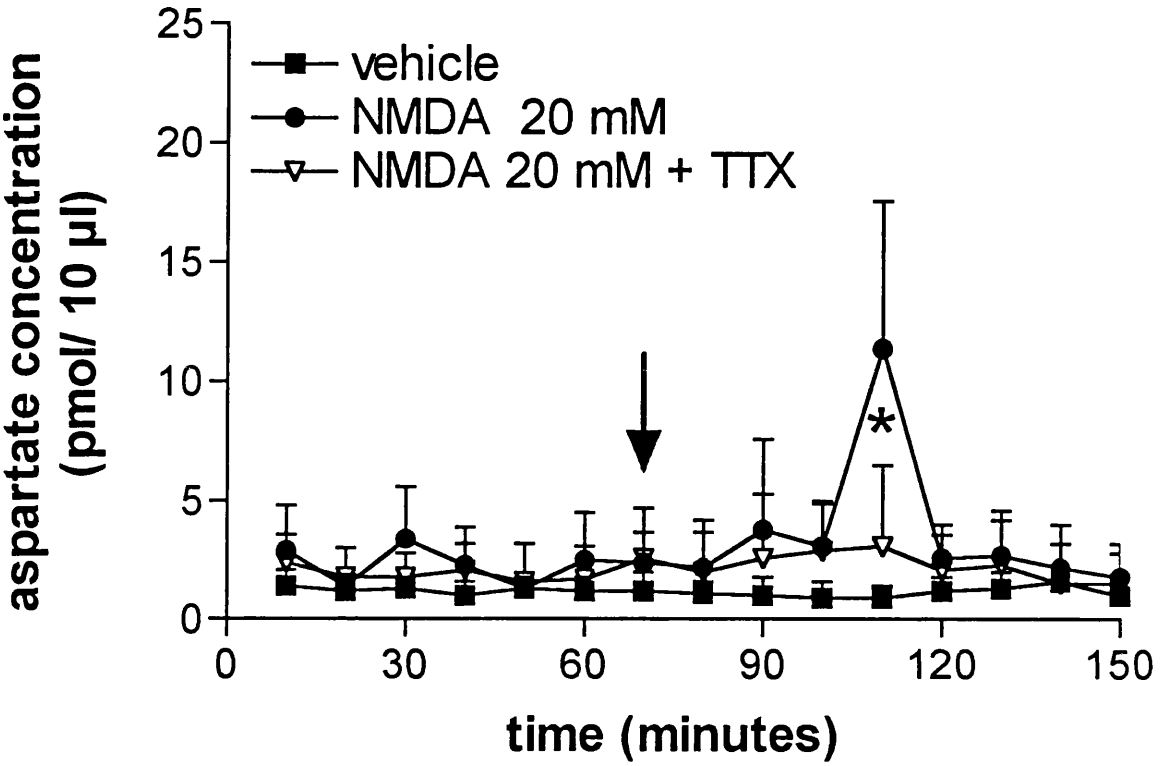
Effect of topical application of NMDA (arrow; 2 mM; n = 7; and 20 mM; n = 6) to the frontal cortex on aspartate concentration in striatal dialysate. * = $P < 0.05$; ** = $P < 0.01$ significantly different from vehicle (Kruskal Wallis ANOVA followed by the Mann-Whitney U-test).

Fig 3,3



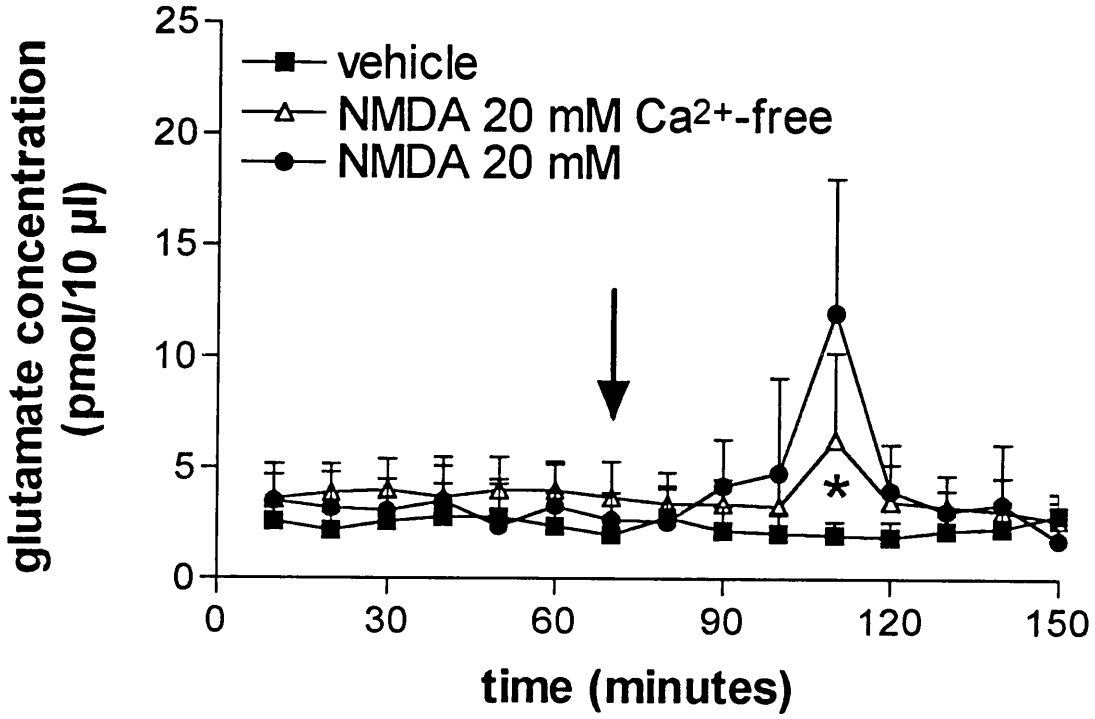
Tetrodotoxin sensitivity of the NMDA (20 mM)-induced increase in glutamate concentration in striatal dialysate. NMDA (arrow, filled squares, n = 6) was coapplied with tetrodotoxin (10 μM, n = 5). * = $P < 0.05$ significantly different from the NMDA effect (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test). Vehicle shown for illustration only.

Fig 3,4



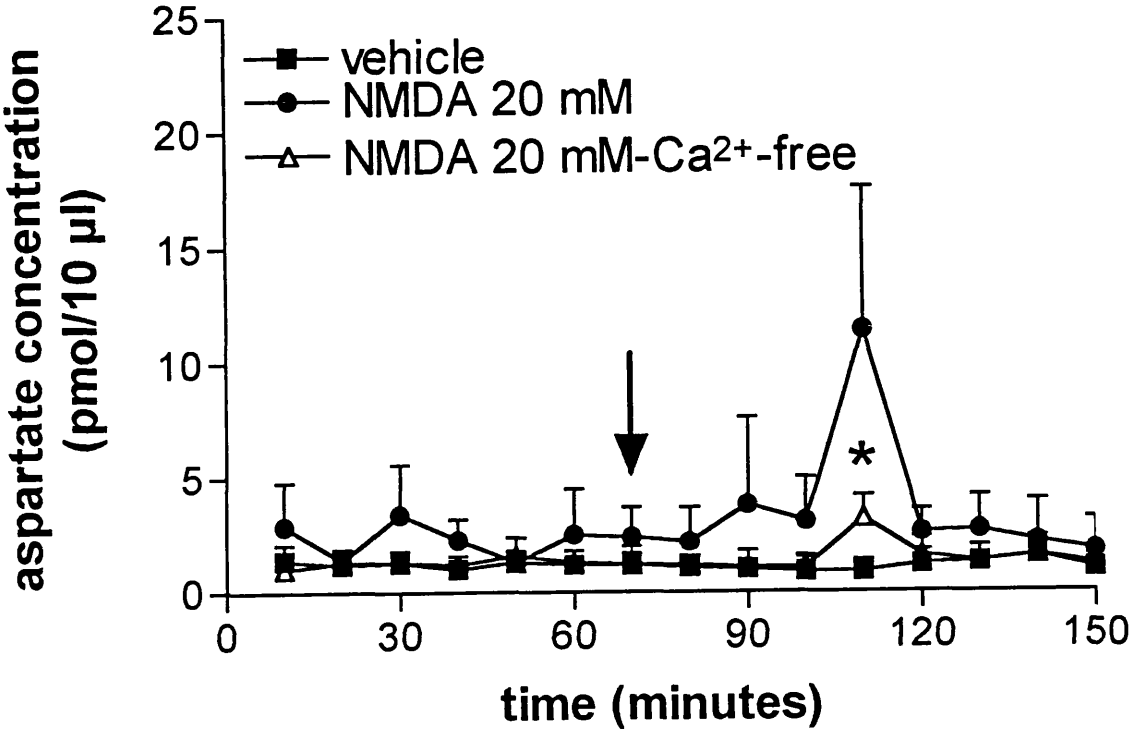
Tetrodotoxin sensitivity of the NMDA (arrow; 20 mM)-induced increase in aspartate concentration in striatal dialysate. NMDA was coapplied with tetrodotoxin (10 μM, n = 5). * = $P < 0.05$ significantly different from the NMDA effect (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 3,5



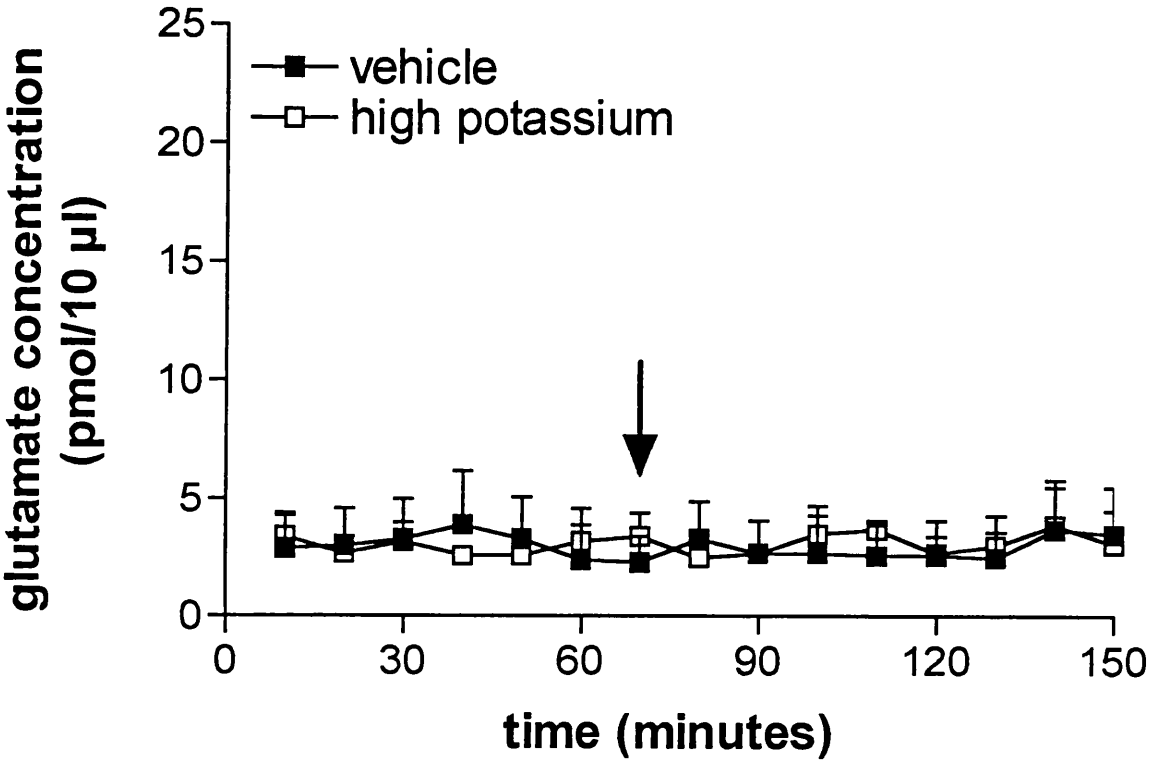
Ca^{2+}-dependency of the NMDA (20 mM, arrow)-induced increase in glutamate concentration in striatal dialysate. Ca^{2+}-free medium (n = 8) was perfused through the dialysis probe from when the probe was implanted in the striatum. * = $P < 0.05$ significantly different from the NMDA effect (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 3,6



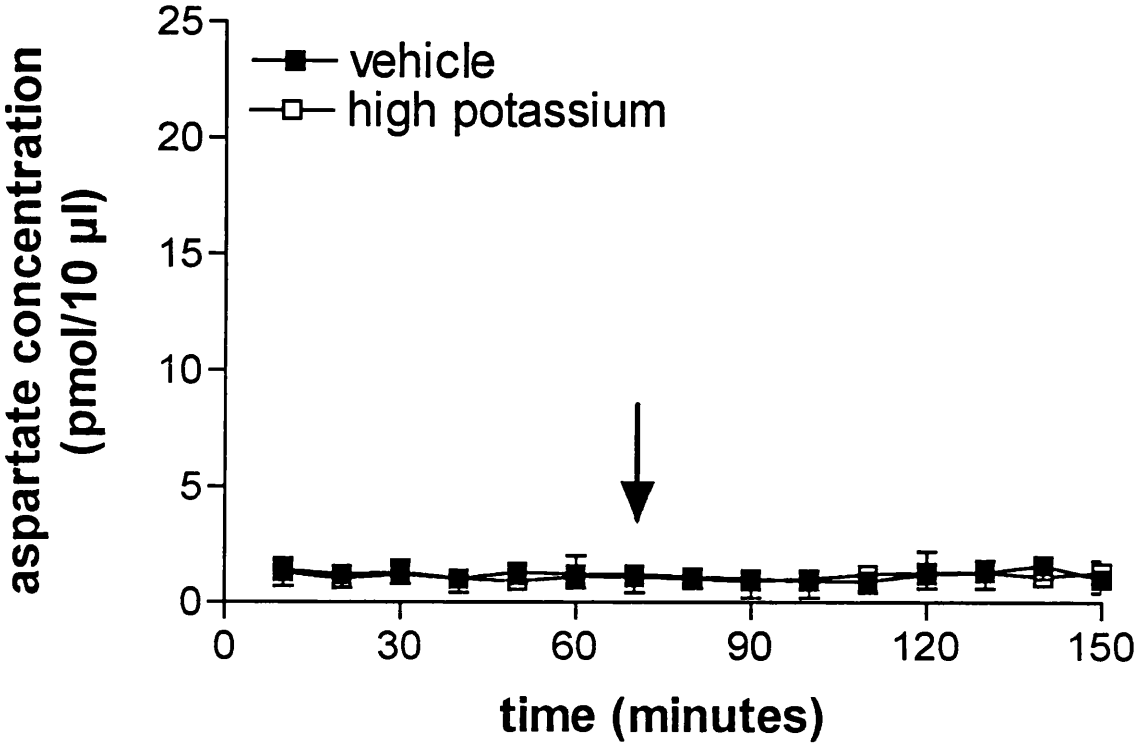
Ca^{2+}-dependency of the NMDA (20 mM, arrow)-induced increase in aspartate concentration in striatal dialysate. Ca^{2+}-free medium (n = 8) was perfused through the dialysis probe from when the probe was implanted in the striatum. * = $P < 0.05$ significantly different from the NMDA effect (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 3,7



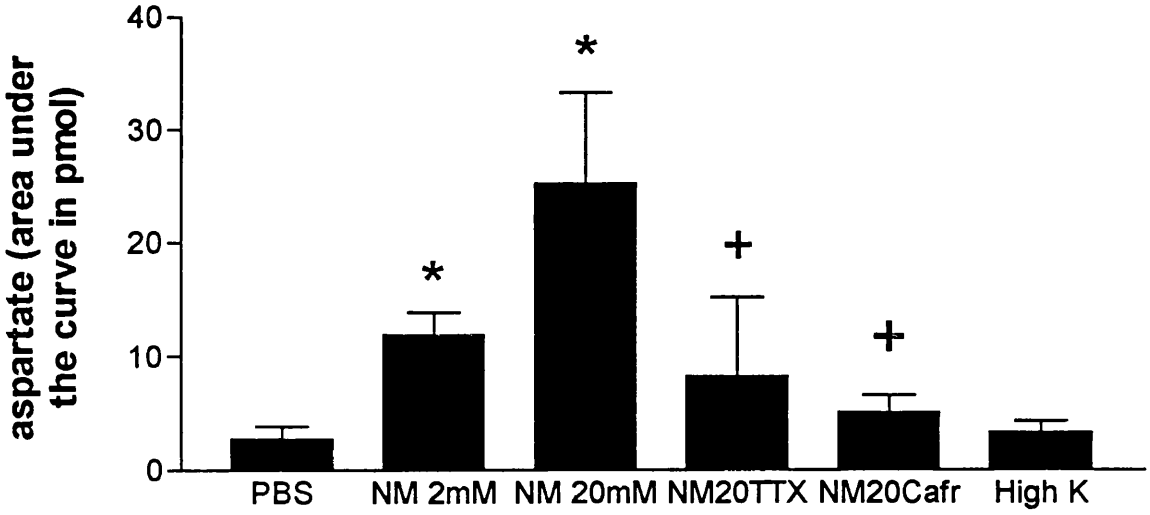
Neither potassium (100 mM; arrow; n = 4), nor vehicle (arrow; n = 8), topically applied to the frontal cortex at the beginning of the seventh sample affect glutamate concentration in striatal dialysate (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test)

Fig 3,8



Neither potassium (100 mM; arrow; n = 4), nor vehicle (arrow; n = 8), topically applied to the frontal cortex affect aspartate concentration in striatal dialysate (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

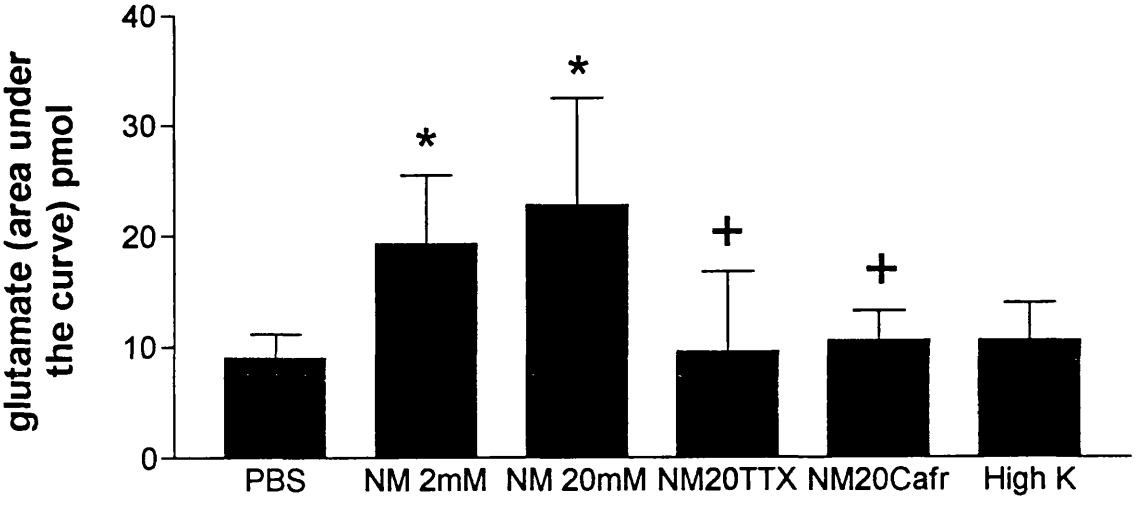
Fig 3,9



Increase in ASP concentration (expressed as area under the curve,pmol) in striatal dialysate after topical application of drugs. Data were analysed using ANOVA followed by either the Mann Whitney U test or the LSD test as appropriate. * = $P < 0.05$ compared to vehicle effect; + = $P < 0.05$, compared to the NMDA effect.

(PBS = phosphate buffered saline; NM 2mM = NMDA 2mM; NM 20mM = NMDA 20mM; NM20TTX = NMDA 20 mM + tetrodotoxin 10 μM; NM20Cafr = NMDA 20 mM, dialysis with Ca^{2+} -free dialysis fluid; High K = 100 mM potassium).

Fig 3,10



Increase in Glu concentration (expressed as area under the curve,pmol) in striatal dialysate after topical application of drugs. Data were analysed using ANOVA followed by either the Mann Whitney U test or the LSD test as appropriate. * = $P < 0.05$ compared to vehicle effect; + = $P < 0.05$, compared to the NMDA effect.

(PBS = phosphate buffered saline; NM 2mM = NMDA 2mM; NM 20mM = NMDA 20mM; NM20TTX = NMDA 20 mM + tetrodotoxin 10 μM; NM20Cafr = NMDA 20 mM, dialysis with Ca^{2+} -free dialysis fluid; High K = 100 mM potassium).

3.5 DISCUSSION

Basal concentrations of the excitatory amino acids glutamate and aspartate, detected with striatal microdialysis and HPLC were similar to those found by Obrenovitch & Richards, (1994), who used similar experimental conditions (same dialysis probe and anaesthesia, same brain area) as those described in this thesis.

The main finding is that topical application of NMDA increased striatal concentrations of both aspartate and glutamate. In a previous study Palmer et al., (1989) observed that glutamate release was only significantly increased when a cocktail of GABA-antagonists was added to NMDA. It seems likely that differences between that study in this one are the result of the methodological changes made.

Differences with the previous study are:

1) A different microdialysis probe was used.

2) The flow rate was reduced from 2 to 1 μl/min.

3) Stabilisation time after implantation of the probe was increased from 0.5 to 1 hour.

4) Different anaesthesia was used (chloral hydrate in the previous study, halothane in a mixture of oxygen and nitrous oxide in this study).

5) A feedback temperature controller was used in the present study, ensuring a constant core temperature during the complete experiment. In the previous study a heating mat was used, with no feedback control.

In view of these factors it is not unexpected that in the present study less variability of the response occurred and that more subtle differences between drug effects can be detected. Also in the present study NMDA released relatively more aspartate than glutamate (20 mM NMDA increased aspartate release by 500 % while it increased glutamate release by 380 %). The reason for this may be that NMDA excessively stimulates the brain region it is topically applied to. That

this is the case is suggested by a study showing that 30 nmoles of NMDA in 3 μl causes extensive damage to the cortex (Palmer et al., 1993). This excessive stimulation may lower extracellular concentrations of glucose, which may be accompanied by a relative increased secretion in ASP over GLU (Szerb & O'Regan, 1987)

It could be argued that application of NMDA to the surface of the cortex would cause a spreading depression of Leao, characterised by loss of cellular ionic homeostasis and a drastic redistribution of ions between the extracellular and intracellular compartments (Leao, 1944), which would then spread through to the deeper layers. Thus, increased release of amino acids could be the result of a nonspecific effect, rather than being the result of an interaction between drugs applied and receptors on the cell bodies of layer V pyramidal neurones. The main reason that this is unlikely is the delay of approximately 30 to 40 min observed between application of drugs and the maximal release of neurotransmitters in the striatum. *In vitro* experiments show that the delay can not be attributed to the dead volume of the microdialysis system. In addition, spreading depression is not mediated by voltage-gated sodium channels (Tobiasz & Nicholson, 1982; Aitken et al., 1991), but in the present study, tetrodotoxin significantly reduced the effect of topically applied drugs. Furthermore a previous study using this paradigm showed that *infusion* of drugs into frontal cortex had a similar effect as topical application of the drugs to the same frontal region (Palmer et al., 1989). Application of 100 mM potassium to the surface of the cortex, did not affect striatal concentrations of either aspartate or glutamate. The rationale behind the experiments with 100 mM K^+ was the difference in diffusibility into brain tissue between NMDA and potassium. While potassium will be quickly taken up both by neurones and glial cells, NMDA will be taken up only at a low rate by low-affinity transport (Skerrit & Johnston, 1981; Garthwaite, 1985). Thus it can be postulated that although both potassium and NMDA will depolarise the surface of the cortex when topically applied, only NMDA will be able to diffuse to layer V of the cortex to directly depolarise the pyramidal neurones which form the corticostriatal pathway. This diffusion will take a certain amount of time, which may explain the

delay seen in the present experiments. The reason why both NMDA and potassium, when topically applied fail to depolarise immediately pyramidal neurones in layer V of the cortex remains unclear. One possibility is that both excitatory and inhibitory neurotransmitters are released with no net effect on layer V.

In conclusion, the most likely reason for the observed effects of the topically applied drugs is that they diffuse through the cortex and affect striatal glutamate and aspartate release when the cell bodies and or dendrites of the neurones in layer V of the cortex are directly depolarised.

Chapter 4

Evidence for modulation of NMDA-induced increase in extracellular concentration of aspartate and glutamate by the 5-HT_{1A} antagonist WAY 100135

4.1 Introduction

The influence of 5-HT on cognition has been the subject of many studies (see for recent review; Sirviö et al., 1994). For example 8-OH-DPAT has been found to impair the memory acquisition in water maze tasks in rats (Hunter, 1989; Carli & Samanin, 1992). The latter study suggests that the effect of this 5-HT_{1A} agonist is not mediated by a decreased serotonin turnover via activation of autoreceptors, but could be due to the activation of postsynaptic receptors. This conclusion was drawn since 5,7-DHT induced serotonin depletion did not prevent, and even potentiated this effect. Drugs which block the effect of serotonin via the 5-HT_{1A} receptor may therefore enhance cognition, an effect which may be mediated through increasing activity of pyramidal neurones.

Several studies have shown a modulatory action of serotonin (5-HT) on the responses of excitatory amino acids in the mammalian nervous system, with both suppressive and enhancing effects reported (McCall & Aghajanian, 1979; Lee et al., 1986; Reynolds et al., 1988). This divergence may be explained by the subtypes of serotonin receptors present on the neurones under study, in that activation of different receptor subtypes could produce either neuronal inhibition or excitation. Other studies indicate that corticostriatal pyramidal neurones of layer V of the rat are enriched with 5-HT_{1A} receptors (Francis et al., 1992b). Pyramidal neurones are hyperpolarised by 5-HT through the 5-HT_{1A} receptor (Andrade & Nicholl, 1987; McCormick & Williamson, 1989).

The working hypothesis of this chapter was therefore that a selective 5-HT_{1A} antagonist would block the hyperpolarising effect of endogenous 5-HT on pyramidal neurones in layer V of the rat cortex, and would potentiate the effect of a depolarising agent such as NMDA.

Until recently only partial agonists of the 5-HT_{1A} receptor were available. However,

recently a selective antagonist, WAY100135 (N-tert-butyl 3-4 (2-methoxyphenyl) piperazin1-yl-2 -phenylpropanamidedihydrochloride) was described. This is an antagonist in both pre and post-synaptic 5-HT_{1A} models and is devoid of agonist properties up to 10 mg/kg. (Fletcher et al., 1993; Routledge et al., 1993). Since the activity of WAY100135 resides in the (+) enantiomer (+) WAY100135 was used for all experiments.

4.2 Materials and Methods

For a detailed description of animals, surgery and HPLC see section 2.1-2.9..

The following drugs (in a volume of 10 μl) were applied to the frontal cortex :

1) NMDA (2 mM), coapplied with WAY 100135 20 μM (n=4), 50 μM (n=13), 100 μM (n=7).
2) NMDA (2 mM), coapplied with 8-OH-DPAT (5 mM ; n=7)
3) NMDA (2 mM), coapplied with WAY 100135 (50 μM) together with tetrodotoxin 10 μM (n=5)
4) NMDA (2 mM) coapplied with WAY 100135 (50 μM), dialysis with Ca^{2+}-free medium (n=7)
5) WAY 100135 topically applied (50 μM, n=10; 100 μM, n=10)
6) WAY 100135 (50 μM) perfused through the probe (n=4)

4.3 Results

4.3.1 glutamate.

Although the mean of the NMDA-induced increase in striatal glutamate was increased by 20 μM WAY 100135 this failed to reach significancy (7.8 $\pm$ 2.3 pmol/10 μl to 10.5 $\pm$ 0.7 pmol/10 μl, $P = 0.0582$, Fig. 4,1). A significant potentiation, however, was induced by 50 μM WAY 100135 in sample 11, (from 7.8 $\pm$ 2.3 pmol/10 μl to 14.3 $\pm$ 6.4 pmol/10 μl, $P = 0.01$, Fig. 4,3), but 100 μM WAY 100135 failed to affect the NMDA signal significantly (for example sample 11, 7.8 $\pm$ 2.3 pmol/10 μl versus 10.1 $\pm$ 7.5 pmol/10 μl, $P = 0.2524$, Fig. 4,5) and this was also a feature of coapplication of 5 mM 8-OH-DPAT (for example sample 11, 7.8 $\pm$ 2.3 pmol/10 μl versus 8.8 $\pm$ 2.2 pmol/10 μl, $P = 0.5$, Fig. 4,7). The increase in glutamate induced by NMDA coapplied with 50 μM WAY 100135 was tetrodotoxin sensitive, the response was reduced in sample 11 (from 14.3 $\pm$ 6.4 pmol/10 μl to 4.2 $\pm$ 1.1, $P < 0.01$), and in sample 12, (5.8 $\pm$ 2.9 pmol/10 μl versus 2.0 $\pm$ 1.2 pmol/10 μl, $P < 0.05$, see Fig. 4,9) The increase in glutamate induced by NMDA coapplied with 50 μM WAY 100135 was Ca^{2+}-dependent (sample 11, reduction from 14.3 $\pm$ 6.4 pmol/10 μl to 6.7 $\pm$ 4.6 pmol/10 μl, $P < 0.01$, see Fig. 4,11).

50 μM WAY 100135 alone, topically applied to the frontal cortex did increase glutamate release in sample 11, (vehicle = 2.6 $\pm$ 1.3 pmol/10 μl versus 3.8 $\pm$ 0.8 pmol/10 μl, $P <$ 0.05; see Fig. 4,13). The increase in glutamate release induced by 100 μM WAY 100135 failed to reach statistical significance (for example sample 11, 2.6 $\pm$ 1.3 pmol/10 μl versus 3.3 $\pm$ 1.6 pmol/10 μl, $P = 0.2524$, see Fig. 4,15).

50 μM WAY 100135, perfused through the dialysis probe from sample number 5-12 did not increase glutamate concentrations in the striatum (see Fig. 4,17).

4.3.2 Aspartate.

The increase in striatal aspartate release induced by 2 mM NMDA (see also 3.1) was potentiated by 20 μM WAY 100135 (topically applied to the frontal cortex) in sample 9, (from 1.7 $\pm$ 0.5 pmol/10 μl to 2.7 $\pm$ 0.1 pmol/10 μl, $P < 0.05$) and in sample 11, from 6.2 $\pm$ 1.0 pmol/10 μl to 9.3 $\pm$ 1.2 pmol/10 μl, $P < 0.05$, see Fig. 4,2). Although 50 μM WAY 100135 increased the mean value of the NMDA-induced effect, this failed to reach significancy (potentiation to 9.3 $\pm$ 4.2 pmol/10 μm, $P = 0.128$, see Fig. 4,4).

In contrast, 100 μM WAY 100135 significantly *reduced* the NMDA-induced effect This reduction was observed in sample 9, (from 1.7 $\pm$ 0.5 pmol/10 μl to 0.6 $\pm$ 0.4 pmol/10 μl, $P < 0.05$), sample 10, (from 2.9 $\pm$ 1.6 pmol/10 μl to 0.7 $\pm$ 0.6 pmol/10 μl, $P < 0.05$) sample 11, (from 6.2 $\pm$ 1.0 pmol/10 μl to 1.4 $\pm$ 0.6 pmol/10 μl, $P < 0.01$) sample 12, (from 2.8 $\pm$ 0.8 pmol/10 μl to 1.0 $\pm$ 0.5 pmol/10 μl, $P < 0.01$, see Fig. 4,6).

The 5-HT_{1A} partial agonist 8-OH-DPAT (5 mM) failed to significantly modulate the NMDA signal in any sample (see Fig. 4,8). The increase in aspartate induced by NMDA coapplied with WAY 100135 (50 μM) was not tetrodotoxin sensitive, (reduction from 9.3 $\pm$ 4.2 pmol/10 μl to 5.2 $\pm$ 3.4 pmol/10 μl, $P = 0.0896$, see Fig. 4,10). The increase in aspartate induced by NMDA coapplied with WAY 100135 (50 μM) was Ca^{2+}-dependent: sample 11, (from 9.3 $\pm$ 4.2 pmol/10 μl to 3.2 $\pm$ 2.0 pmol/10 μl, $P < 0.01$), sample 12, (from 4.9 $\pm$ 3.1 pmol/10 μl to 1.3 $\pm$ 0.3 pmol/10 μl, $P < 0.01$), and sample 13, (3.1 $\pm$ 2.0 pmol/10 μl versus 1.4 $\pm$ 0.4 pmol/10 μl, $P < 0.05$, see Fig. 4,12).

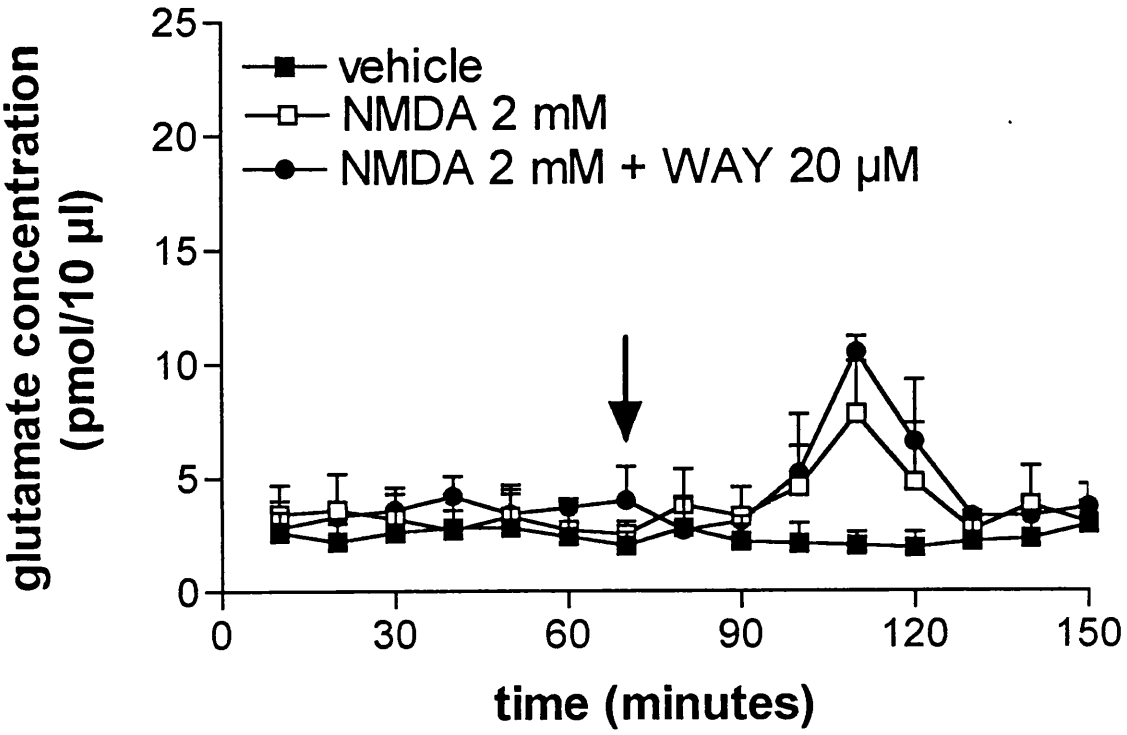
WAY 100135 (50 μM), topically applied to the frontal cortex did not increase aspartate release (vehicle = 0.8 $\pm$ 0.4 pmol/10 μl versus WAY 100135 1.0 $\pm$ 0.6 pmol/10 μl, $P = 0.2786$, see Fig. 4,14). Similarly, WAY 100135 (100 μM) did not have an effect on basal aspartate release in sample 11 (vehicle = 0.8 $\pm$ 0.4 pmol/10 μl versus 1.1 $\pm$ 0.6 pmol/10 μl, $P = 0.141$, see Fig. 4,16).

50 μM WAY 100135 when perfused through the dialysis probe from sample number 5-12 did not significantly affect basal aspartate release (see Fig. 4,18).

4.3.3 Aspartate and Glutamate area under the curve and ASP/GLU ratio.

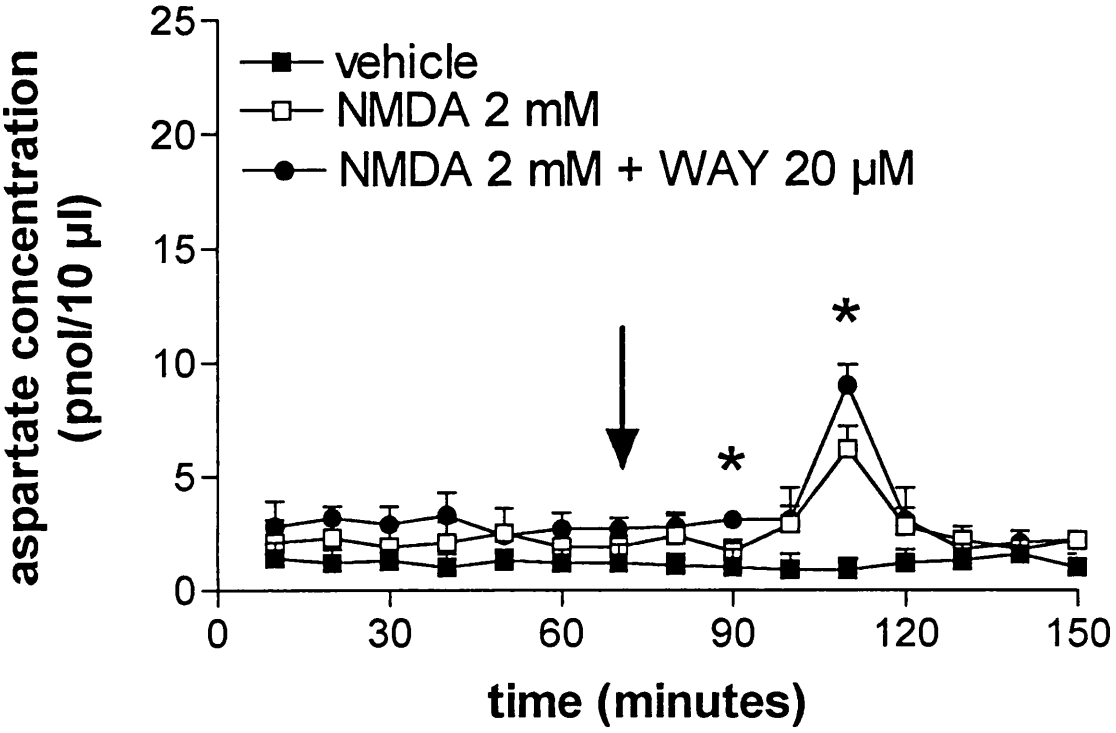
Treatment	area under curve ASP	area under curve GLU	ratio ASP/GLU
vehicle	2.7	9.0	0.3
NMDA 2mM	11.9	19.3	0.6
NM +WY20	16.8	16.3	1.03
NM + WY50	17.5	27.7	0.6
NM +WY100	3.0	13.3	0.2
NM +DPAT	8.8	16.2	0.5
WY 50	2.9	11.9	0.24
WY 100	3.7	11.6	0.3
NM +WY +TTX	9.4	9.9	0.9
NM +WY +Ca^{2+}-free	5.7	11.8	0.5

Fig 4,1



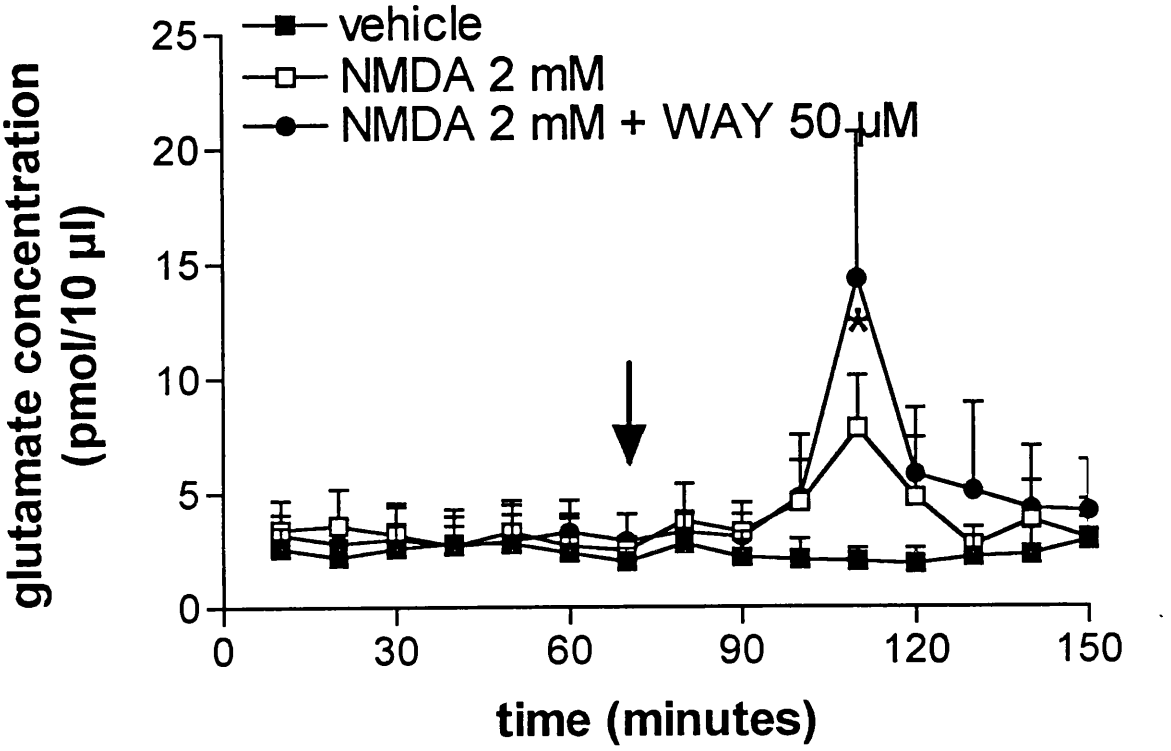
Effect of WAY 100135 (20 μM, arrow, n = 4) , coapplied with NMDA (2 mM; n = 7), on glutamate concentration in striatal dialysate. No significant difference between the NMDA effect and the effect of the combination of drugs was observed. Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test.

Fig 4,2



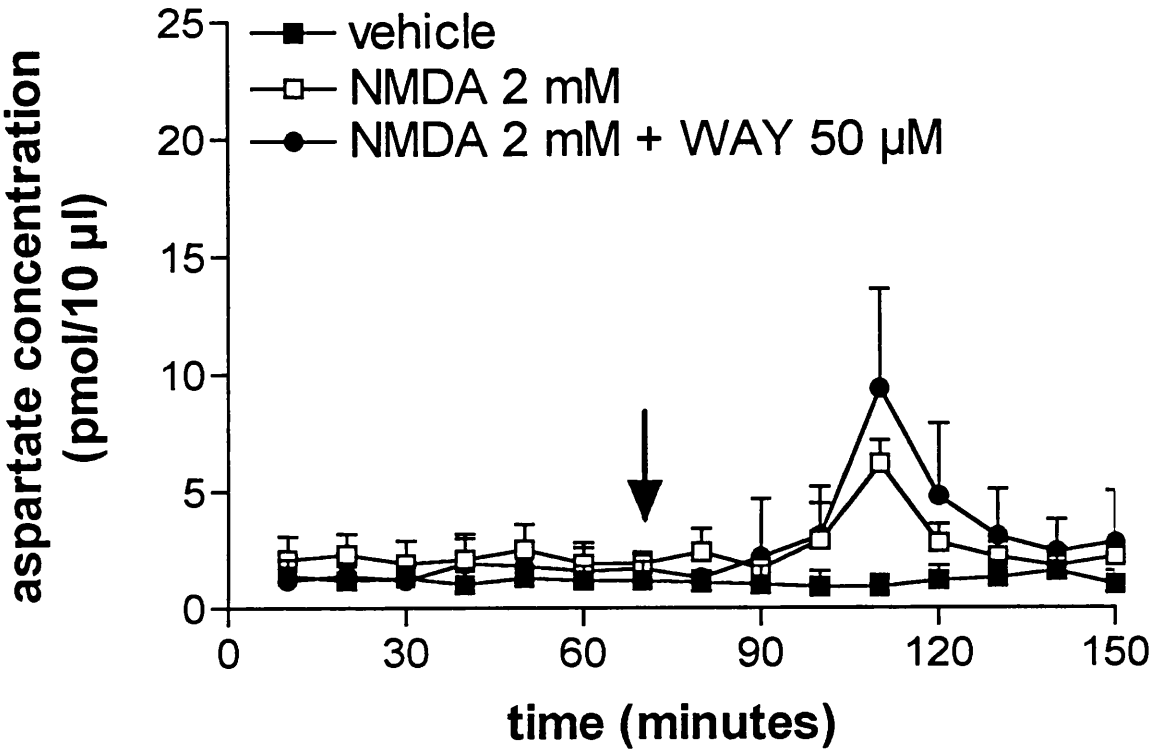
Effect of WAY 100135 (20 μM, arrow, n = 4), coapplied with NMDA (2 mM; n=7), on aspartate concentration in striatal dialysate. A significant difference between the NMDA effect and the effect of the combination of drugs was observed. * = $P < 0.05$ (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test.

Fig 4,3



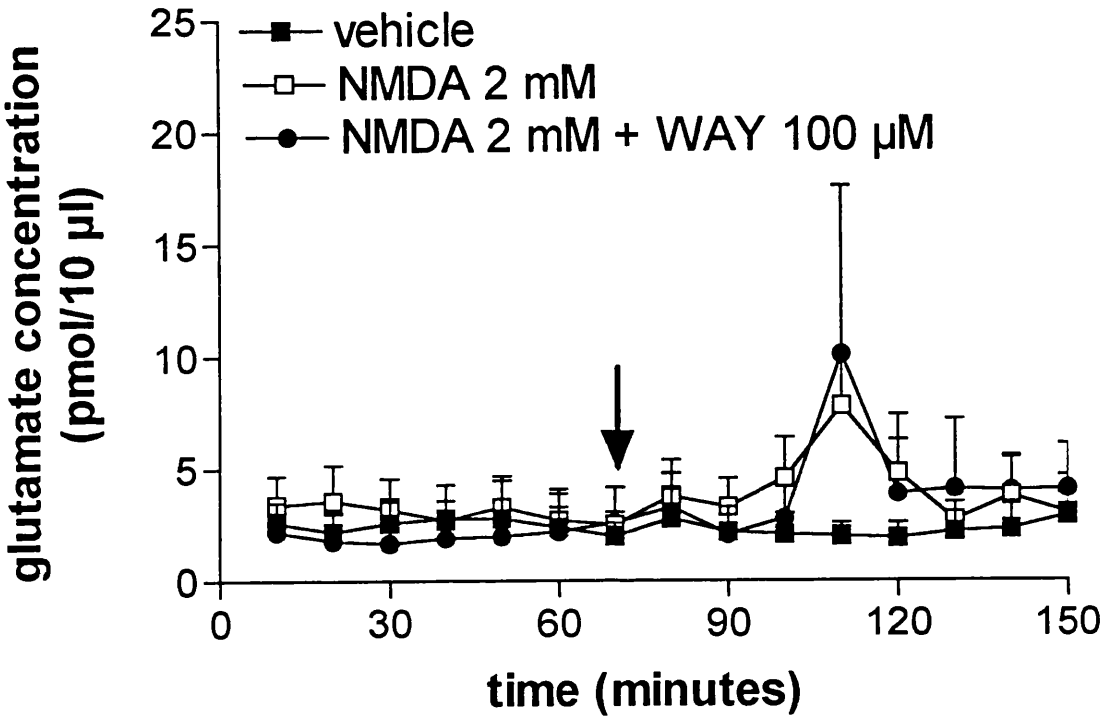
Effect of WAY 100135 (50 μM, arrow, n = 13) , coapplied with NMDA (2 mM, n = 7), on glutamate concentration in striatal dialysate. A significant difference between the NMDA effect and the effect of the combination of drugs was observed. * = $P < 0.05$. (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test.

Fig 4,4



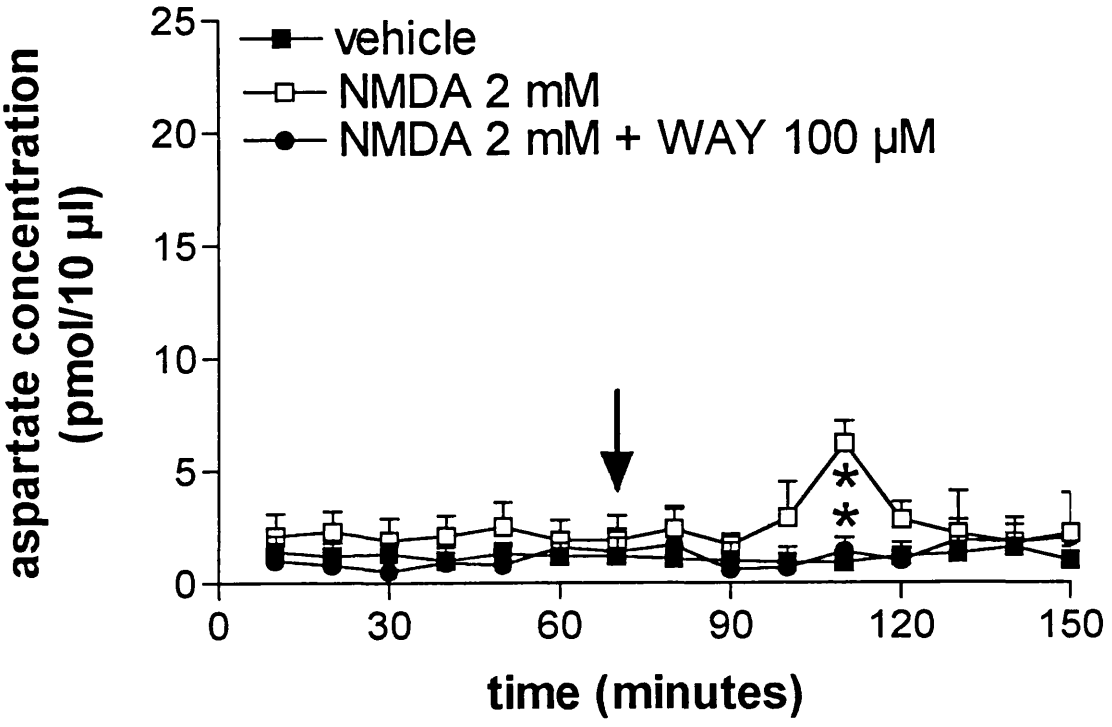
Effect of WAY 100135 (50 μM, arrow, n = 13), coapplied with NMDA (2 mM, n = 7), on aspartate concentration in striatal dialysate. Although the mean of the NMDA signal was increased, this failed to reach statistical significance (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,5



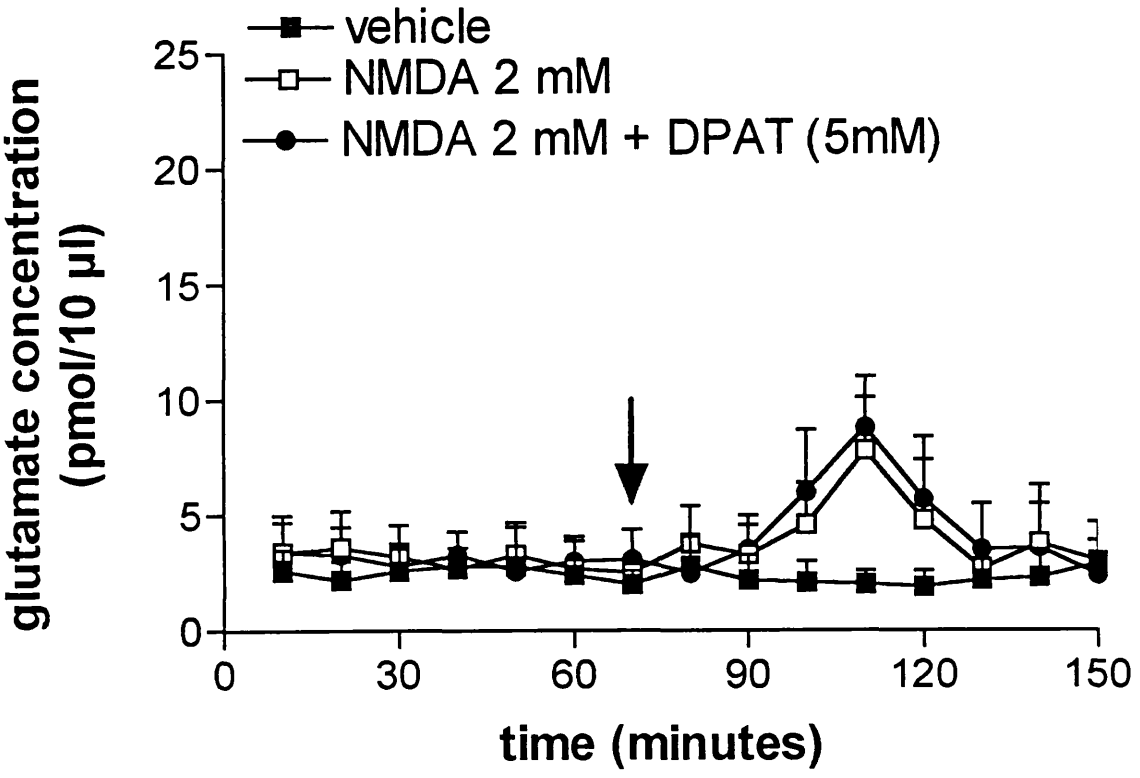
Effect of WAY 100135 (100 μM, arrow, n = 7) , coapplied with NMDA (2 mM, n = 7), on glutamate concentration in striatal dialysate. No significant effect of the NMDA effect by coapplied WAY 100135 was noted (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,6



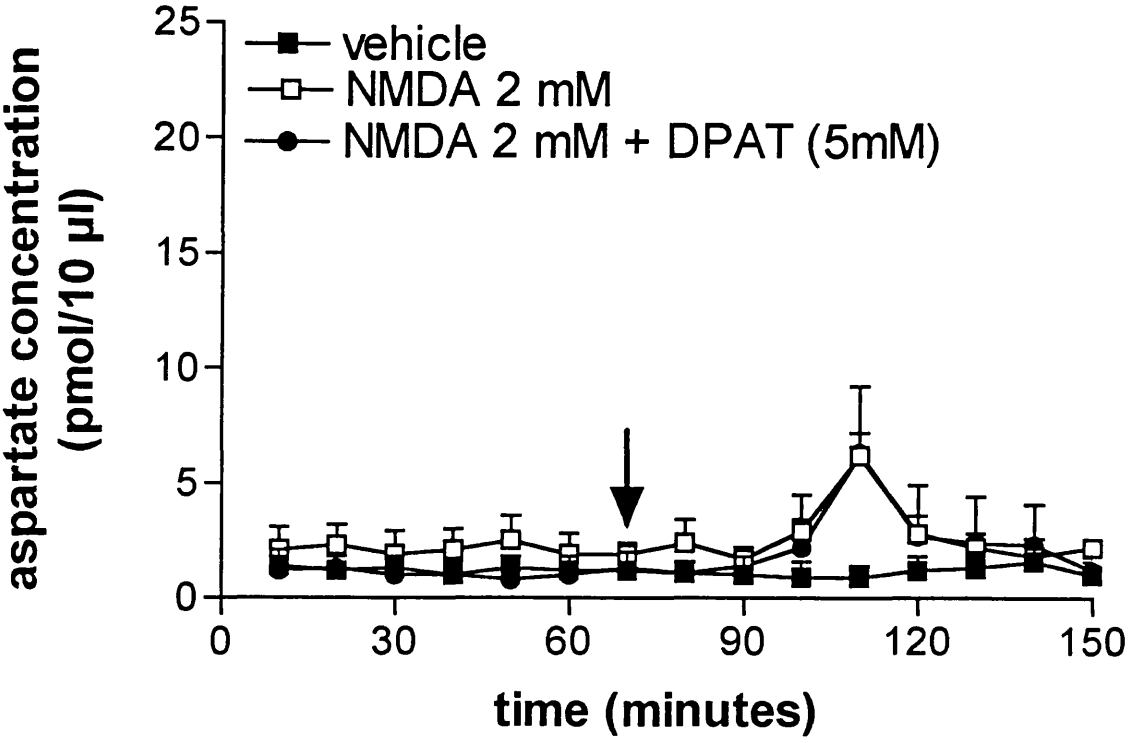
Effect of WAY 100135 (100 μM, arrow, n = 7) , coapplied with NMDA (2 mM, n = 7), on aspartate concentration in striatal dialysate. A significant reduction of the NMDA effect by coapplied WAY 100135 was noted. * = $P < 0.05$; ** = $P < 0.01$ (Kruskal-Wallis ANOVA followed by the Mann- Whitney-U test.

Fig 4,7



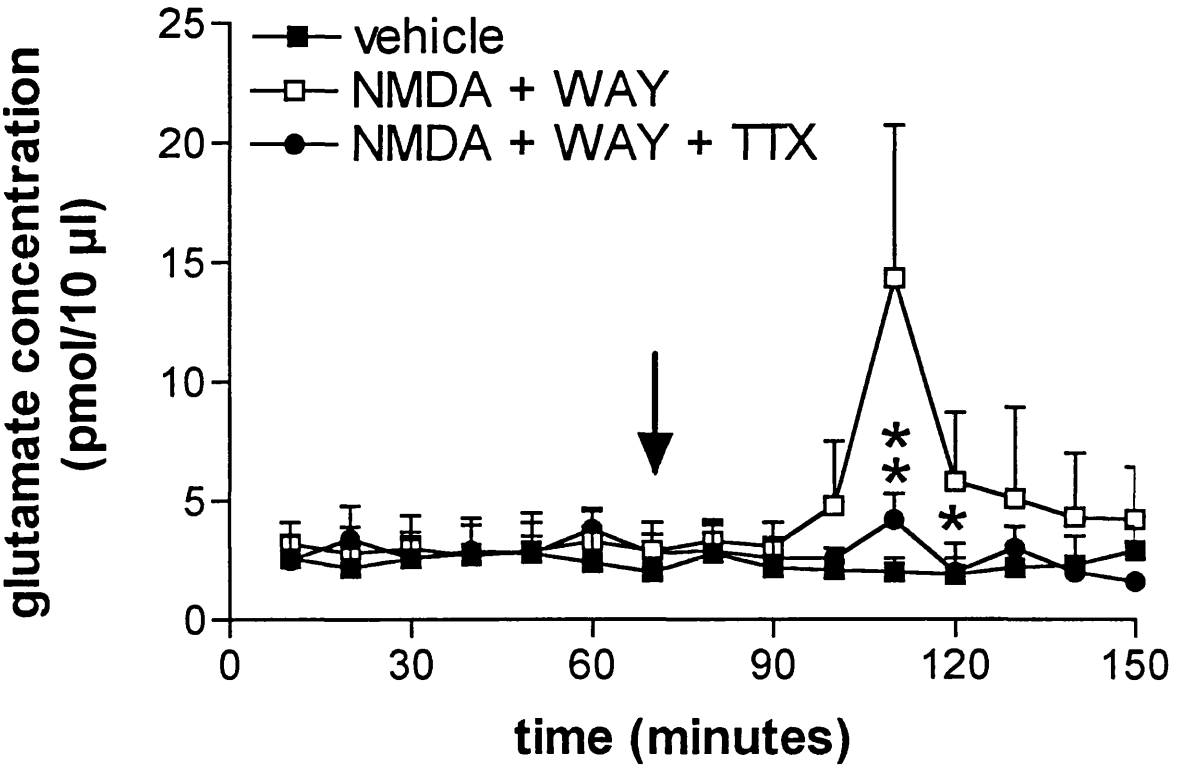
Effect of 8-OH-DPAT (5 mM, arrow, n = 7) , coapplied with NMDA (2 mM, n = 7), on glutamate concentration in striatal dialysate. No significant difference between the NMDA effect and the effect of the combination of drugs was noted (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,8



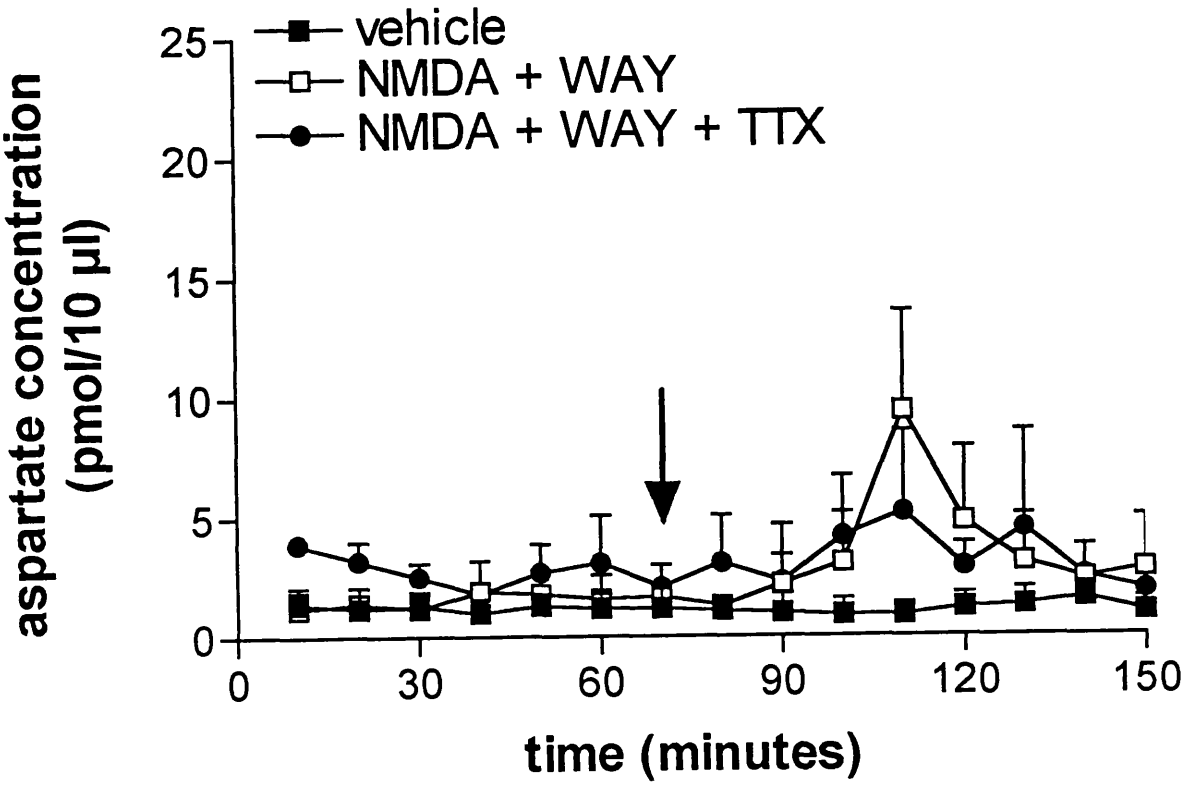
Effect of 8-OH-DPAT (5 mM, arrow, n = 7) , coapplied with NMDA (2 mM, n = 7), on aspartate concentration in striatal dialysate. No significant difference between the NMDA effect and the effect of the combination of drugs was noted (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,9



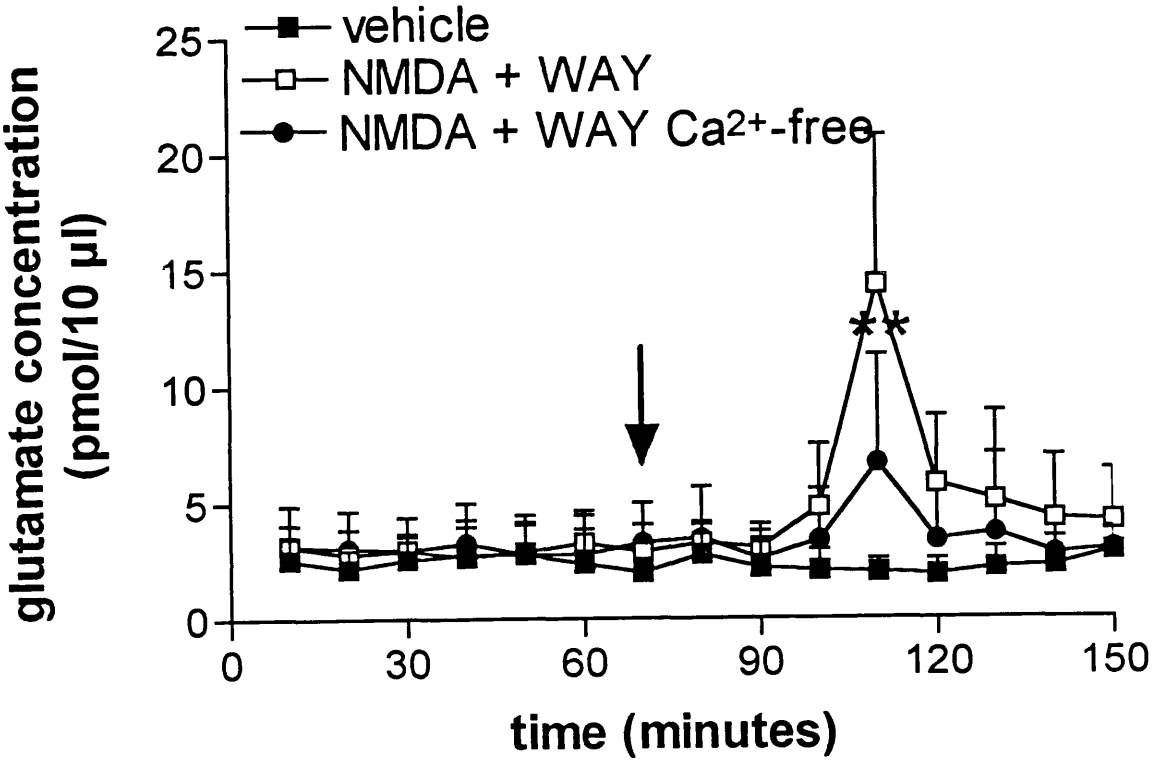
Tetrodotoxin sensitivity (10 μM, n = 5) of the effect on glutamate concentration in striatal dialysate of the combination of 2 mM NMDA with 50 μM WAY 100135. A significant reduction of NMDA-induced glutamate release was observed. * = $P < 0.05$, ** = $P < 0.01$ (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,10



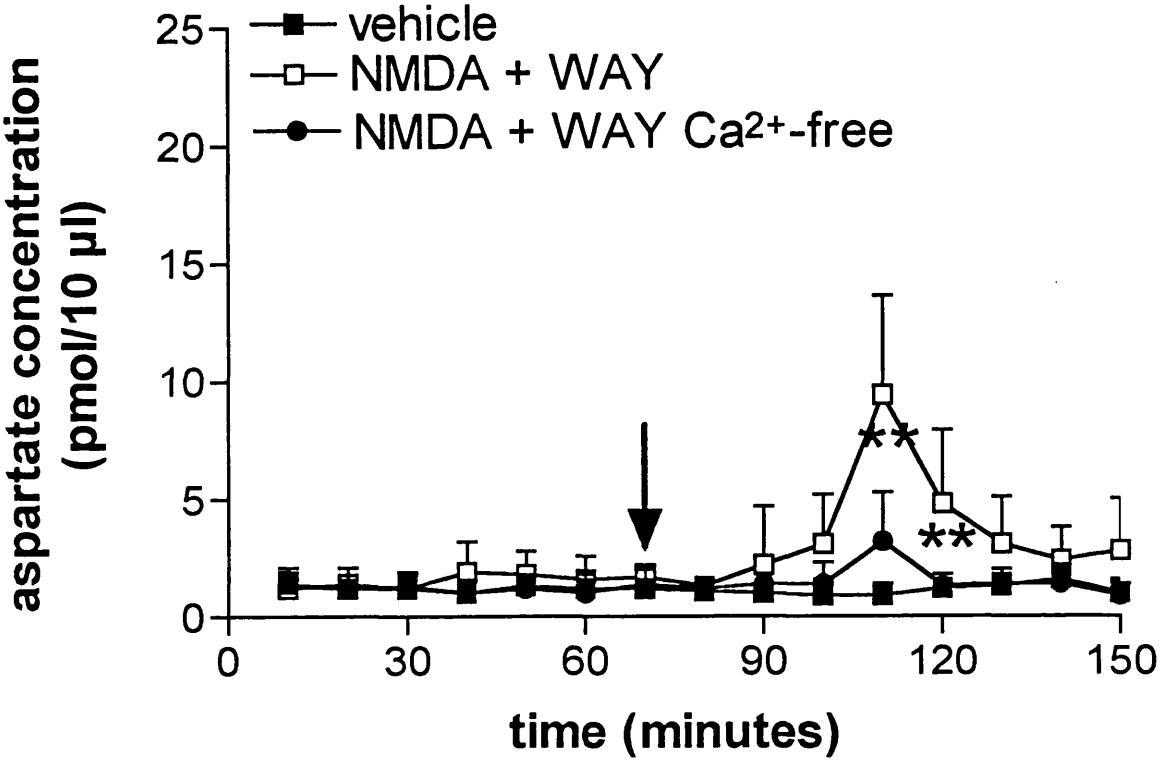
Tetrodotoxin sensitivity (10 μM, n = 5) of the effect on aspartate concentration in striatal dialysate of the combination of 2 mM NMDA with 50 μM WAY 100135. Although the mean was reduced this did not reach statistical significance (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,11



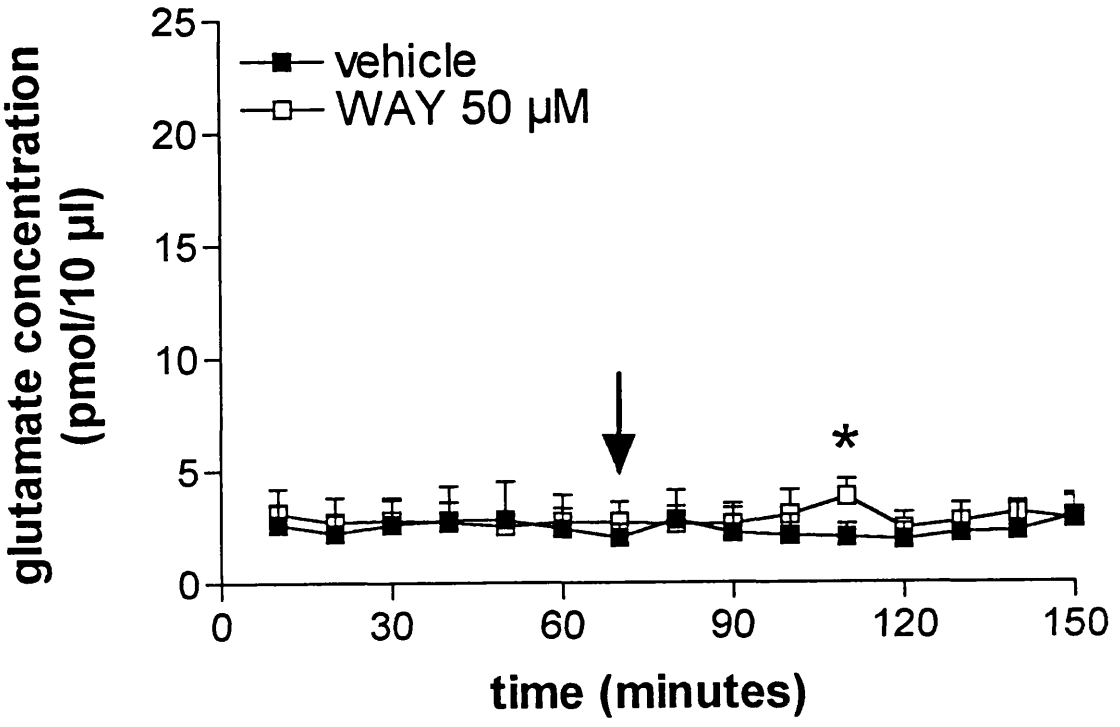
Ca^{2+}-dependency (n = 7) of 2 mM NMDA coapplied with 50 μM WAY 100135-induced increase in glutamate concentration in striatal dialysate. Ca^{2+}-free perfusion fluid was perfused through the probe from the moment of implantation. ** = $P < 0.01$ (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,12



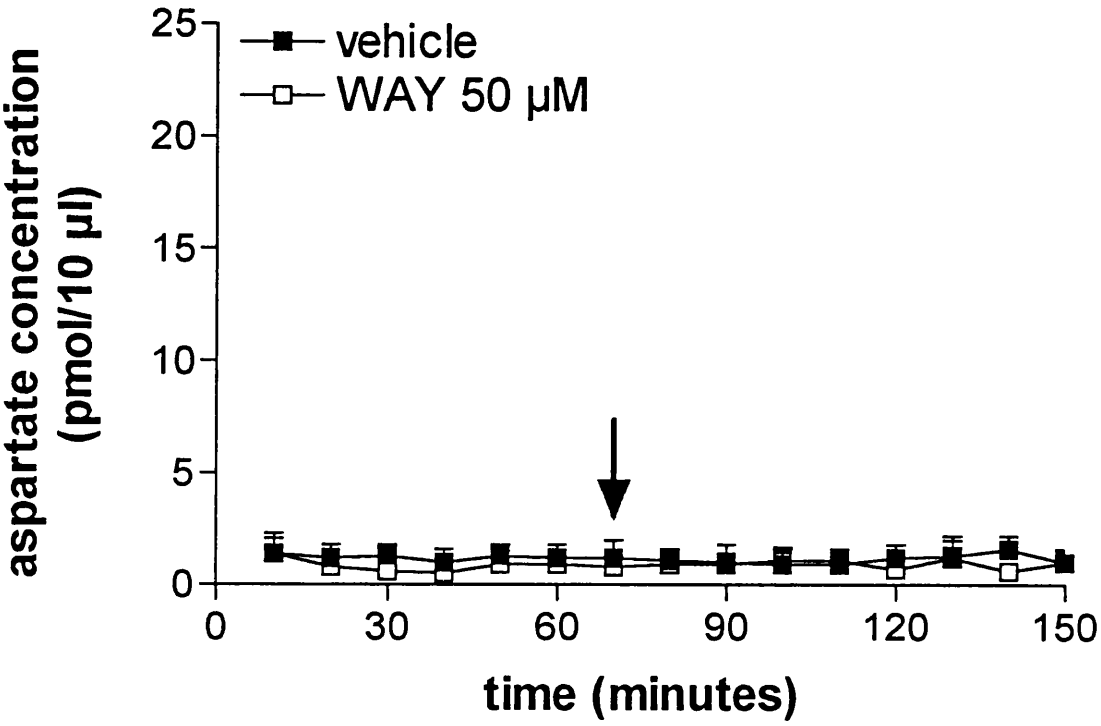
Ca^{2+}-dependency (n = 7) of 2 mM NMDA coapplied with 50 μM WAY 100135-induced increase in aspartate concentration in striatal dialysate. Ca^{2+}-free perfusion fluid was perfused through the probe from the moment of implantation. * = $P < 0.05$, ** = $P < 0.01$ (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,13



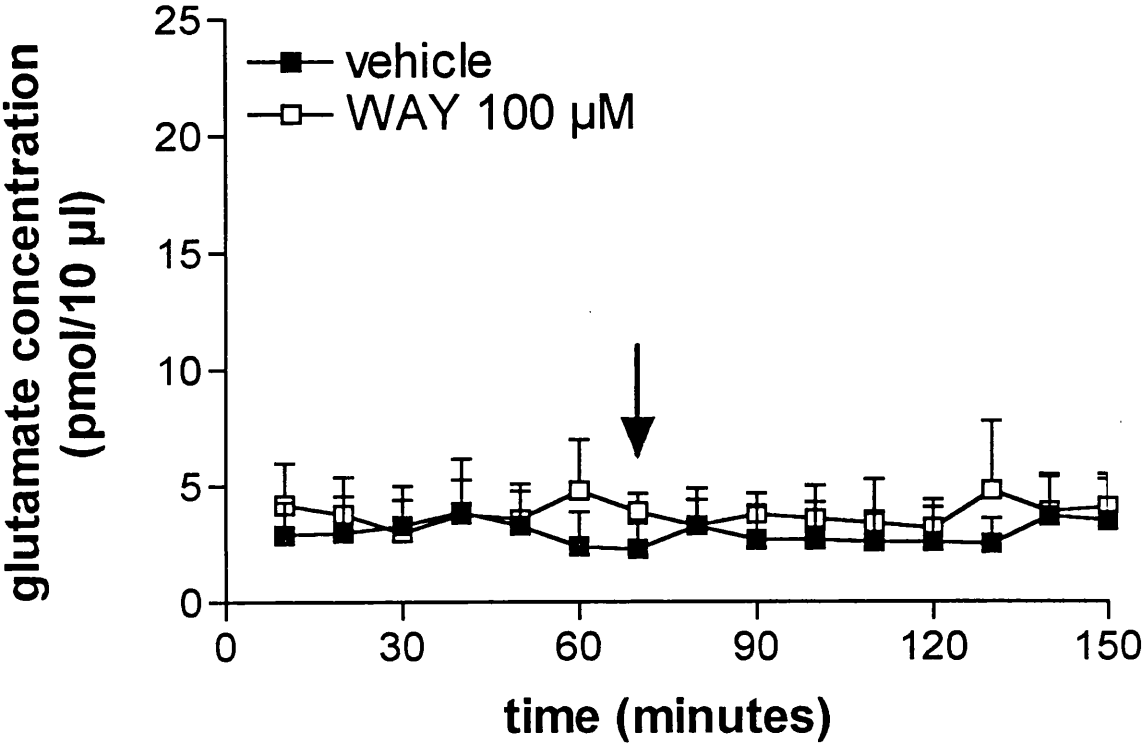
Effect of WAY 100135 (arrow, 50 μM, n = 10), topically applied to the frontal cortex, on glutamate concentration in striatal dialysate. A significant increase was detected in the eleventh sample, as compared to basal values. * = $P < 0.05$ (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,14



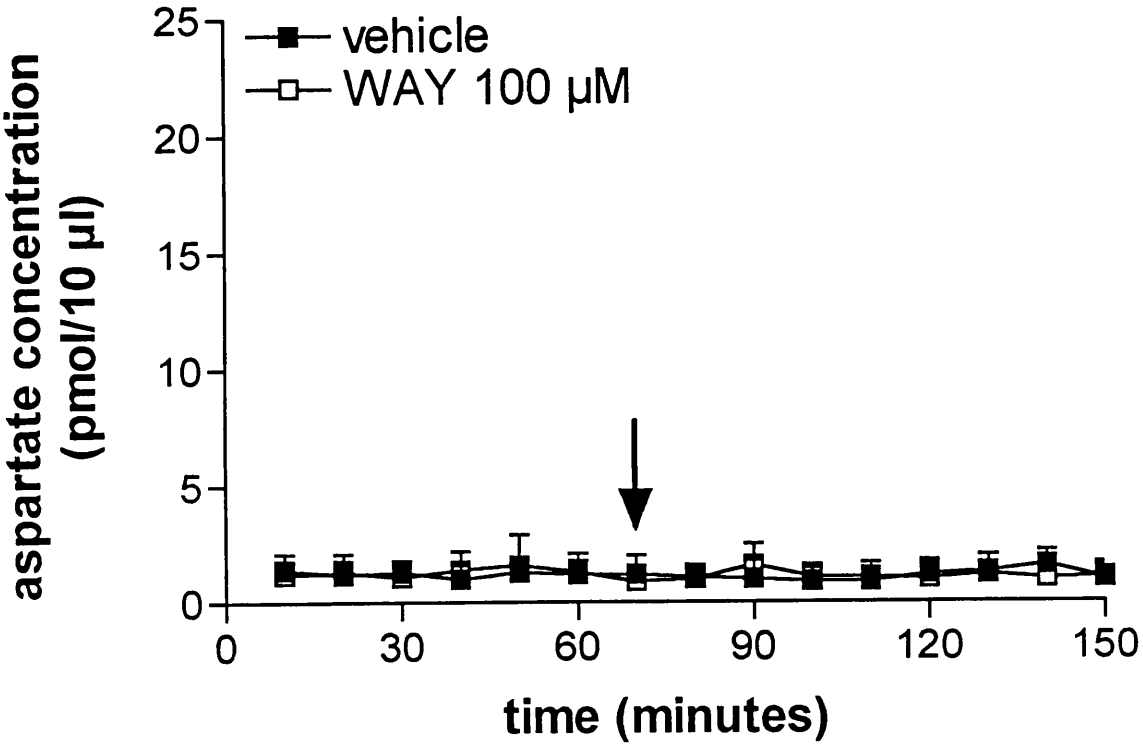
Effect of WAY 100135 (arrow, 50 μM, n = 10), topically applied to the frontal cortex, on aspartate concentration in striatal dialysate. No significant increase was detected in any sample, compared to basal values (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,15



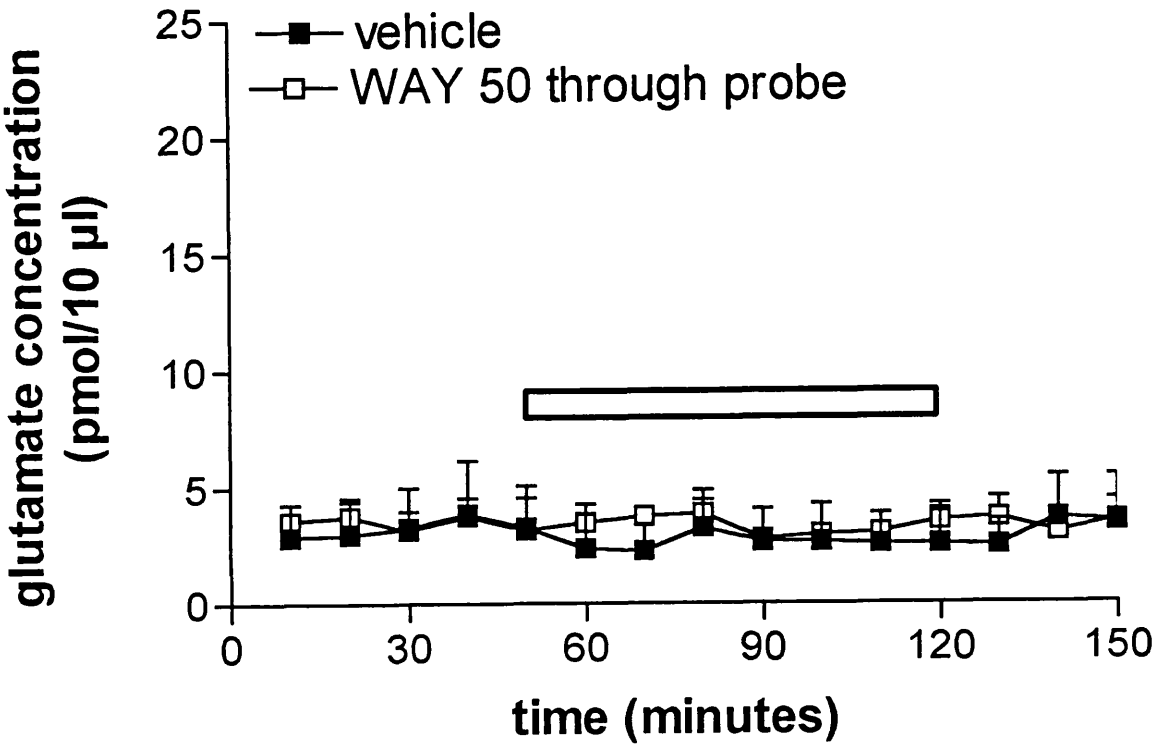
Effect of WAY 100135 (arrow, 100 μM, n = 10), topically applied to the frontal cortex, on glutamate concentration in striatal dialysate. No significant increase was detected in any sample, as compared to basal values (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,16



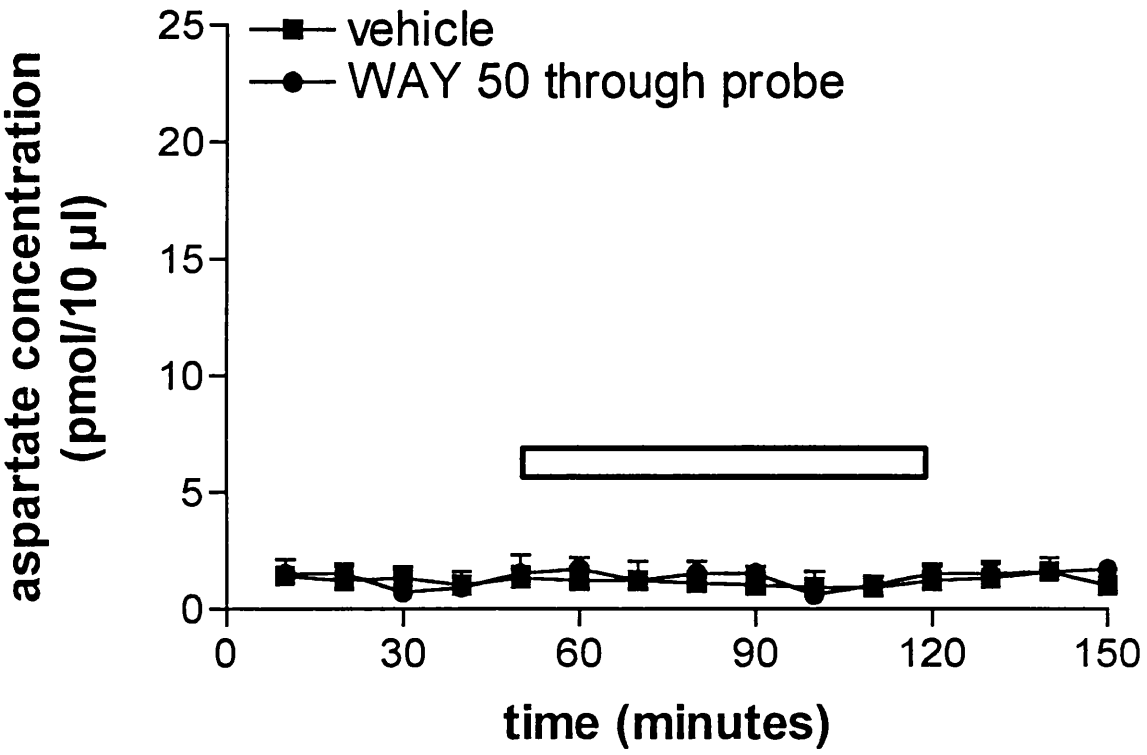
Effect of WAY 100135 (arrow, 100 μM, n = 10), topically applied to the frontal cortex, on aspartate concentration in striatal dialysate. No significant increase was detected in any sample, as compared to basal values (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,17



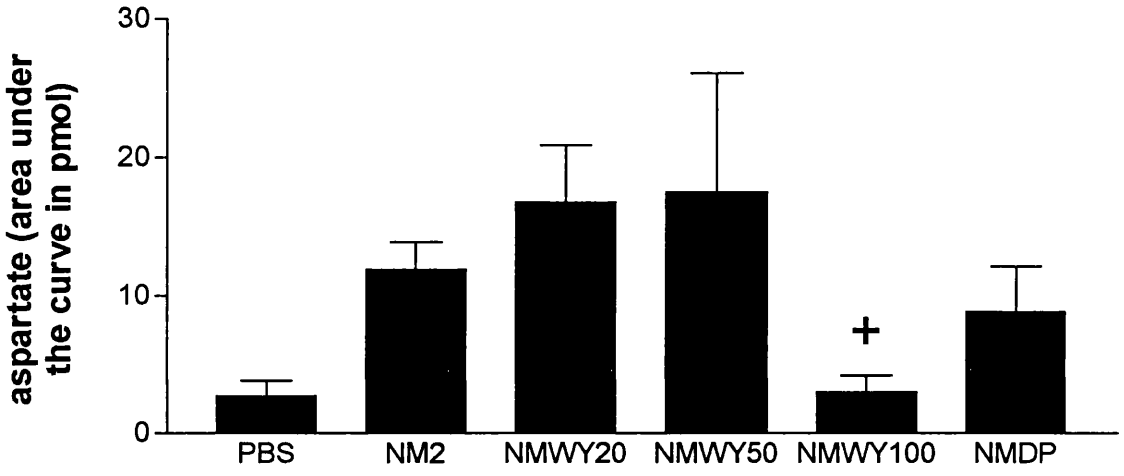
Effect of WAY 100135, perfused through the dialysis probe (50 μM, bar, n = 4) on glutamate concentration in striatal dialysate. No significant increase was detected in any of the samples, as compared to basal values (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

Fig 4,18



Effect of WAY 100135, perfused through the dialysis probe (50 μM, bar, n = 4) on aspartate concentration in striatal dialysate. No significant increase was detected in any of the samples, as compared to basal values (Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test).

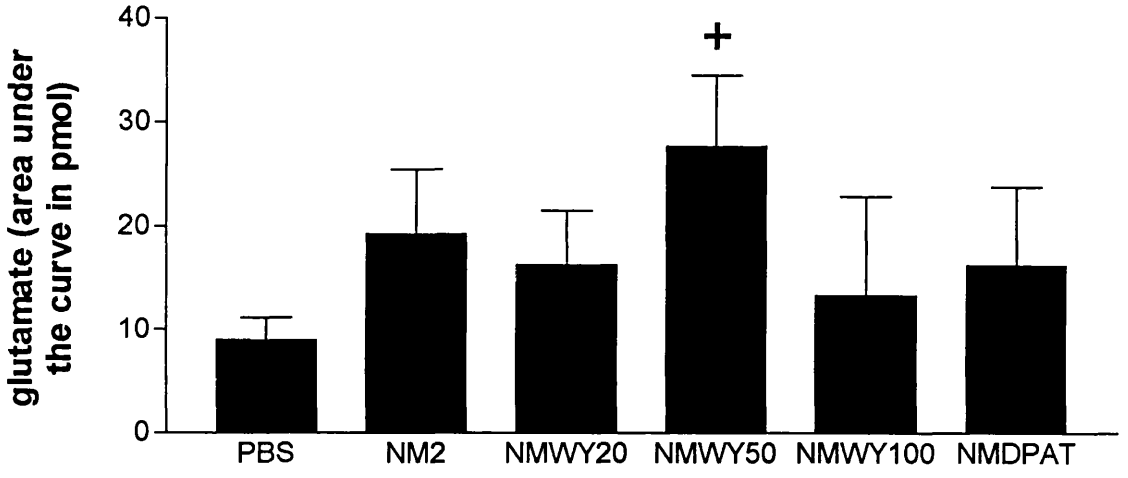
Fig 4,19



Effect of drugs, topically applied to the frontal cortex, on concentration of ASP in striatal dialysate, expressed as area under the curve (pmol).Data were analysed using Kruskal-Wallis ANOVA, followed by either a Mann Whitney U test or a LSD test, as appropriate. + = $P < 0.05$, difference with NMDA effect.

(PBS = phosphate buffered saline; NM2 = NMDA 2 mM; NMWY20 = NMDA 2mM + WAY 100135 20 μM; NMWY50 = NMDA 2 mM + WAY 100135 50 μM; NMWY100 = NMDA 2mM +WAY 100135 100 μM; NMDP = NMDA 2 mM + 8-OH-DPAT 5 mM)

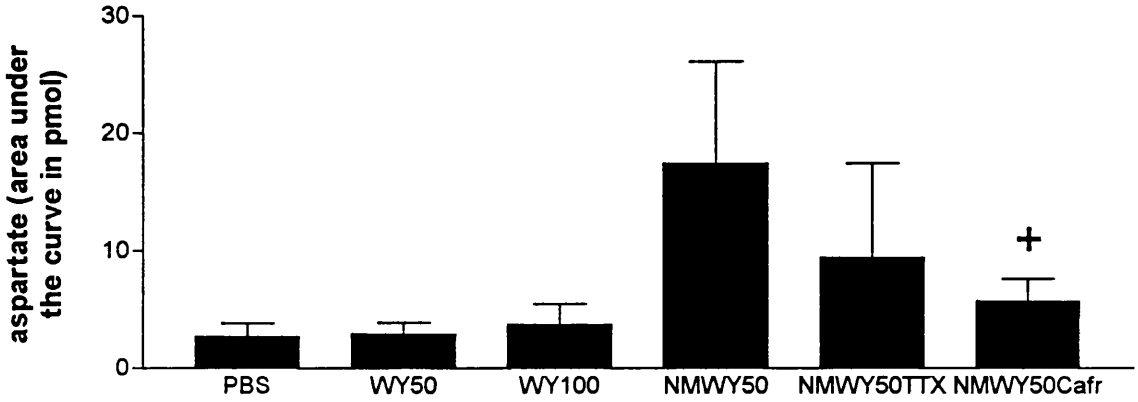
Fig 4,20



Effect of drugs, topically applied to the frontal cortex, on concentration of GLU in striatal dialysate, expressed as area under the curve (pmol).Data were analysed using Kruskal-Wallis ANOVA, followed by either a Mann Whitney U test or a LSD test, as appropriate. + = $P < 0.05$, difference with NMDA effect.

(PBS = phosphate buffered saline; NM2 = NMDA 2 mM; NMWY20 = NMDA 2mM + WAY 100135 20 μM; NMWY50 = NMDA 2 mM + WAY 100135 50 μM; NMWY100 = NMDA 2mM +WAY 100135 100 μM; NMDP = NMDA 2 mM + 8-OH-DPAT 5 mM)

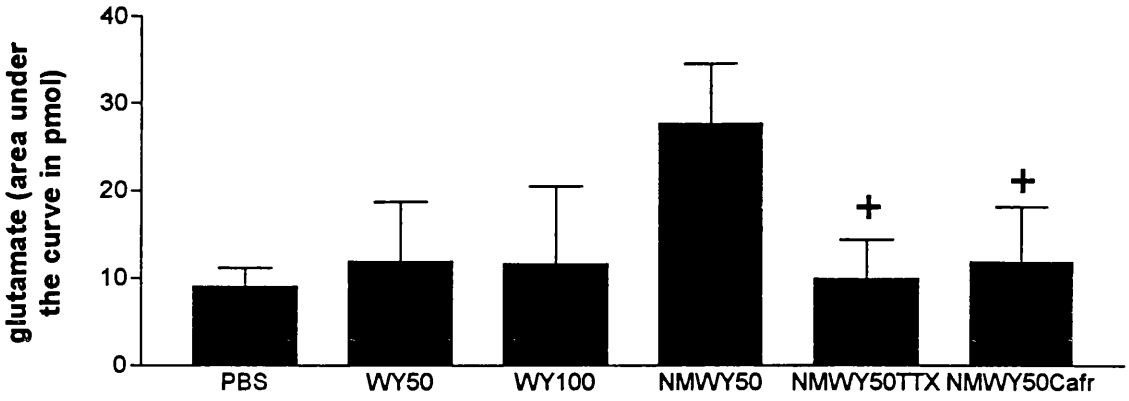
Fig 4,21



Effect of drugs, topically applied to the frontal cortex, on concentration of ASP in striatal dialysate, expressed as area under the curve (pmol).Data were analysed using Kruskal-Wallis ANOVA, followed by either a Mann Whitney U test or a LSD test, as appropriate. + = $P < 0.05$, difference with effect of NMDA + WAY.

(PBS = phosphate buffered saline; WY50 = WAY 100135 50 μM; WY100 = WAY 100135 100 μM; NMWY50 = NMDA 2 mM + WAY 100135 50 μM; NMWY50TTX = NMDA 2 mM + WAY 100135 50 μM + tetrodotoxin 10 μM; NMWY50Cafr = NMDA 2 mM + WAY 100135 50 μM, dialysis with Ca^{2+}-free fluid).

Fig 4,22



Effect of drugs, topically applied to the frontal cortex, on concentration of GLU in striatal dialysate, expressed as area under the curve (pmol).Data were analysed using Kruskal-Wallis ANOVA, followed by either a Mann Whitney U test or a LSD test, as appropriate. + = $P < 0.05$, difference with effect of NMDA + WAY.

(PBS = phosphate buffered saline; WY50 = WAY 100135 50 μM; WY100 = WAY 100135 100 μM; NMWY50 = NMDA 2 mM + WAY 100135 50 μM; NMWY50TTX = NMDA 2 mM + WAY 100135 50 μM + tetrodotoxin 10 μM; NMWY50Cafr = NMDA 2 mM + WAY 100135 50 μM, dialysis with Ca^{2+}-free fluid).

4.4 Discussion

NMDA-induced release of glutamate and aspartate was potentiated by the 5-HT_{1A} antagonist WAY 100135, although at different concentrations. The most straightforward explanation for this effect is that the compound reduces the resting potential of cortical pyramidal neurones, by blocking the action of 5-HT, which can be a hyperpolarising transmitter, thereby increasing the likelihood that a given cell is depolarised by NMDA. Pioneering (Krnjevic & Phillis, 1963), and more recent (Andrade & Nicholl, 1987; Colino & Halliwell, 1987; Araneda & Andrade, 1991) electrophysiological studies suggests that 5-HT can inhibit cortical pyramidal neurones in layer V of the cortex, a hypothesis supported by this study. The characterisation of WAY 100135 as a 5-HT_{1A} antagonist has been detailed in the paper by Fletcher et al., (1993). The compound shows selective antagonism at postsynaptic 5-HT_{1A} receptors. The IC_{50} of (±) WAY 100135 at the rat hippocampal 5-HT_{1A} receptor was 34 nM, whereas its IC_{50} at a range of other receptor sites was $> 2\ \mu M$. Up to a dose of 2.5 mg/kg 1.v. (±) WAY 100135 induced a maximum of 30 % inhibition of dorsal raphe neuronal firing and (at 0.5 mg/kg i.v.) antagonised the inhibition of firing induced by 8-OH-DPAT, in anaesthetised rats. (±) WAY 100135 antagonised the action of 5-carboxamidoiodotrypamine in the guinea-pig ileum, with a pA_2 of 7.2. The compound had no agonist-like behavioural effects by antagonised the behavioural syndrome and hypothermia induced by 8-OH-DPAT in the rat and the mouse respectively. The interaction of the compound with the 5-HT_{1A} receptor was stereoselective; the (+) enantiomer being much more active in binding, functional and behavioural studies.

That WAY 100135 acts as an antagonist under the experimental conditions presented in this thesis, is suggested by the results obtained with the 5-HT_{1A} agonist 8-OH-DPAT, which failed to affect the NMDA response. This could be due to the fact that endogenous levels of 5-HT saturate the endogenous serotonergic modulation. Under these circumstances the effects of WAY 100135 cannot be ascribed to an agonist effect.

An unexpected divergence in the response of aspartate and glutamate to WAY 100135 was clearly evident. Although the effect of the lower concentration of NMDA on both aspartate and glutamate was potentiated by WAY 100135, the maximum potentiation of aspartate release was at a lower concentration of the drug (20 μM) than for the effect on glutamate (50 μM). Similarly, WAY 100135 (100 μM) did not alter NMDA induced glutamate release but significantly attenuated NMDA induced aspartate release compared to NMDA alone. There is no clear explanation for the divergent effects of WAY 100135 on aspartate and glutamate release. One possibility is that the corticostriatal pathway consists of at least two types of neurone, one using preferentially aspartate as a transmitter the other, glutamate. Such neurone populations may have 5-HT_{1A} receptors which are differently coupled to an effector system. This could result in a different pharmacological profile, an idea consistent with the detection of three mRNAs in the mammalian brain, suggesting the existence of subtypes of 5-HT_{1A} receptors (Albert et al., 1990). Alternatively, there may be a greater enrichment of 5-HT_{1A} receptors on putative aspartergic compared with glutamatergic neurones.

Chapter 5

Evidence that physostigmine increases glutamate but not aspartate concentration in the striatum through the M_1 receptor

5.1 Introduction

In vitro studies have suggested that ACh produces a prolonged facilitation of cortical neurones, rendering them more likely to depolarise following excitatory glutamatergic input (see for recent review: McCormick, 1992). To test this hypothesis in an *in vivo* setting the topical application model has been used in combination with intramuscular injection of the acetylcholinesterase inhibitor physostigmine. The origin of part of the corticostriatal pathway is thought to be in layer V of the cortex. Using striatal concentrations of glutamate and aspartate as putative parameters of activity the effect of i.m. physostigmine, under conditions shown to increase ACh in the frontal cortex (Messamore et al., 1993) has been investigated. In addition, the effect of increasing local ACh concentrations in the striatum was investigated by adding physostigmine to the perfusion fluid at a concentration which has been shown to increase ACh in the striatum (Xiao et al., 1993).

In order to provide more evidence that ACh positively modulates activity of cortical pyramidal neurones *in vivo* via the M_1 receptor, two experiments were performed: a) PD142505-0028, (1-Azabicyclo [2.2.1] heptan-3-one, O-[3-(3-methoxyphenyl)-2-propynyl]oxime, (Z)-(=+/)-, ethanedioate (1:1) (salt) (Schwarz et al., 1994) a selective M_1 partial agonist, was topically applied to the frontal cortex: b) The M_1 antagonist telenzepine dihydrochloride (Kawashima et al., 1990) was topically applied to establish whether this would block the effect of i.m. physostigmine. These drugs were chosen because previous studies indicate that the M_1 receptor is enriched on cortical pyramidal neurones in layers III and V of the rat (Chessell et al., 1993; Chessell et al., 1994). Thus, corticostriatal and corticocortical pyramidal neurones are likely to be subject to similar cholinergic regulation (see also McCormick & Williamson, 1989).

5.2 Materials and methods

The following series of experiments were performed:

1) Intramuscular physostigmine injection (0.3 mg/kg, beginning of the second collection period; n=7)
2) intramuscular vehicle injection (n=7)
3) Physostigmine perfused through the dialysis probe (10 μM, n=6)
4) Intramuscular physostigmine injection combined with tetrodotoxin topically applied to the frontal cortex(10 μM; n=6)
5) Intramuscular physostigmine injection, dialysis with Ca^{2+}-free medium (n=7)
6) Intramuscular physostigmine injection combined with telenzepine, topically applied to the frontal cortex (5 μM; n=6)
7) PD 142505-0028, topically applied to the frontal cortex (10 μM, n=6)

5.3 Statistical analysis and presentation of data.

Data are Mean $\pm$ SD, and expressed in pmol amino acids/10 μl dialysate. An unbalanced repeated measures ANOVA (module 5v; BMDP, Cork, Ireland) was used to test the effect of treatment and the interaction between treatment and time. Individual time points were then compared using one-way ANOVA and the LSD or Student's t test as appropriate.

5.4 Results

5.4.1 Glutamate.

Physostigmine (0.3 mg/kg i.m. at the beginning of the second collection period) significantly increased striatal glutamate concentrations. There was a significant effect of treatment ($F = 6.83$, $df = 3,25$, $P < 0.01$), no overall effect of sample number ($F = 2.14$, $df = 12,3$) but a significant interaction ($F = 3.19$, $df = 36,3$, $P < 0.01$). The physostigmine-induced increase in dialysate glutamate started in the fifth sample (vehicle, 3.2 ± 0.5 pmol/10 μl versus $6.7 \pm$ 2.6pmol/10 μl, $P < 0.05$), and the maximal response was observed in the ninth sample (vehicle, 3.6 ± 0.7 pmol/10 μl versus physostigmine, 8.3 ± 3.3 pmol/10 μl, $P < 0.05$, see Fig. 5,1).

Tetrodotoxin (10 μM) was topically applied at the beginning of collections periods 6-15 in physostigmine-treated rats. This reduced the physostigmine-induced increase in dialysate glutamate in samples 7-14, except sample 10. ($F = 1.481$, $df = 1,10$; $F = 2.027$, $df = 14,140$ and $F = 1.344$, $df = 14,140$ for treatment, sample number and interaction respectively, $P < 0.05$, Student's t-test, see Fig. 5,3).

The physostigmine-induced increase in dialysate concentrations of glutamate was Ca^{2+}-dependent, leading to significant reductions (Student's t-test, $P < 0.05$) in samples 1, 5, 6, 7, 8, 9, 10 and 11 ($F = 14.26$, $df = 1,11$; $F = 1.89$, $df = 14,154$; $F = 1.84$, $df = 14,154$ for treatment, sample number and interaction respectively, see Fig. 5,5).

Telenzepine significantly ($P < 0.05$, ANOVA, LSD) reduced the physostigmine induced increase in glutamate in sample 6, 7, 8, 9, 11 and 13. A significant effect of treatment was observed ($F = 8.94$, $df = 1,11$, $P < 0.05$), no significant sample effect ($F = 1.04$, $df = 14$, $P > 0.05$), but a significant interaction ($F = 2.0$, $df = 14,182$, $P < 0.05$, see Fig. 5,7).

Physostigmine perfused through the dialysis probe did not significantly increase striatal

glutamate concentrations ($P > 0.05$, ANOVA, LSD, see Fig. 5,9)

The M_1 partial agonist PD 142505-0028, topically applied to the frontal cortex, immediately induced an increase in glutamate concentrations in dialysate. There were significant effects of treatment ($F = 16.98$, $df = 1,14$, $P < 0.01$), sample number ($F = 4.65$, $df = 8,112$, $P < 0.01$) and interaction ($F = 4.69$, $df = 8,112$, $P < 0.05$). The significant effect of PD 142505-0028 on glutamate concentrations started in sample 7 and lasted until sample 11 (ANOVA, Student's t-test, $P < 0.05$, see Fig. 5,11)

5.4.2 Aspartate.

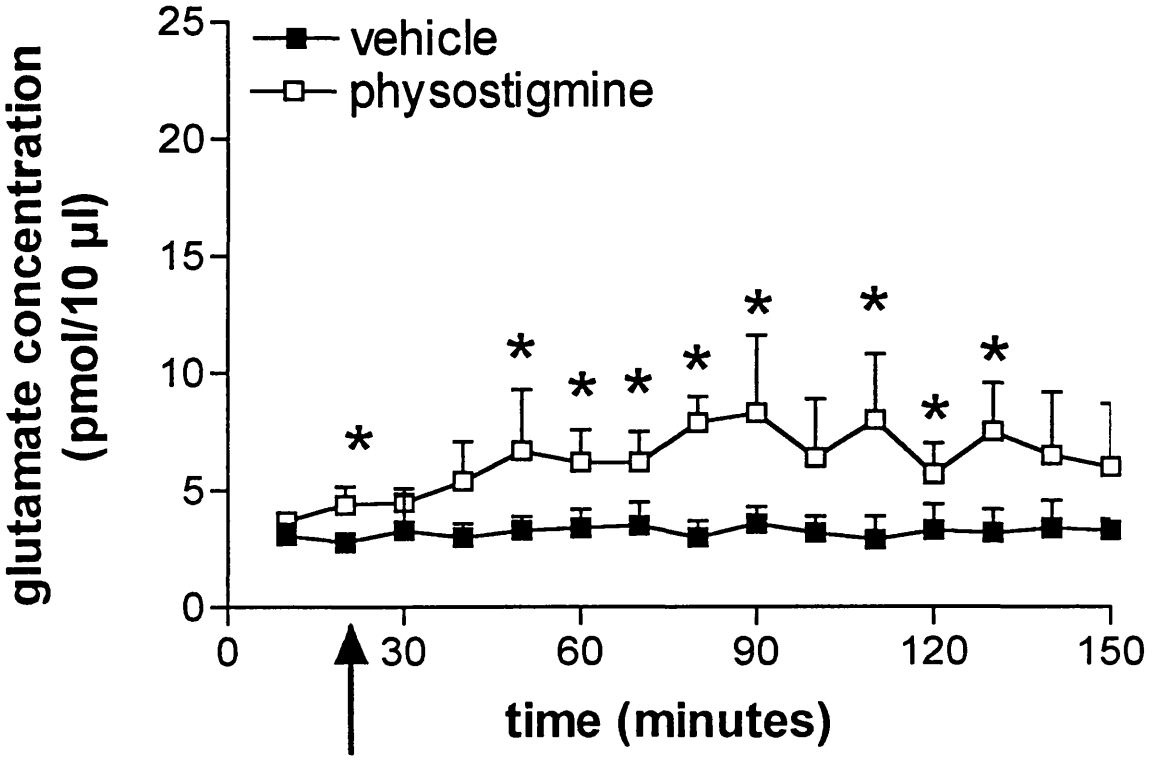
Neither physostigmine (0.3 mg/kg i.m. at the beginning of the second collection period) nor PD 142505-0028 significantly increased aspartate concentration in striatal dialysate (as compared to vehicle)

See Figures 5,2; 5,4; 5,6; 5,8; 5,10 and 5,12.

5.4.3 Glutamate area under the curve

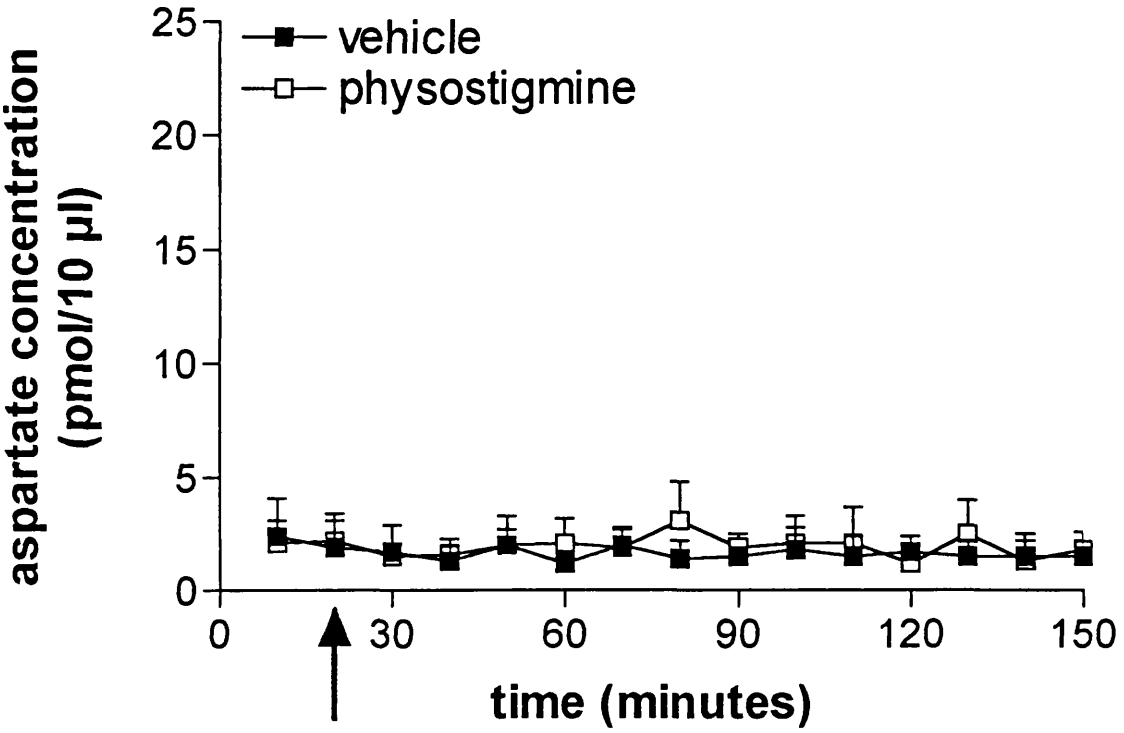
Treatment	area under the curve GLU
PBS	45.7
PHYS	61.8
PHYS +TEL	31
PHYS + TTX	45.9
PHYS Ca^{2+}-free	33.2
PD	13.3
PBS	8.4

Fig 5,1



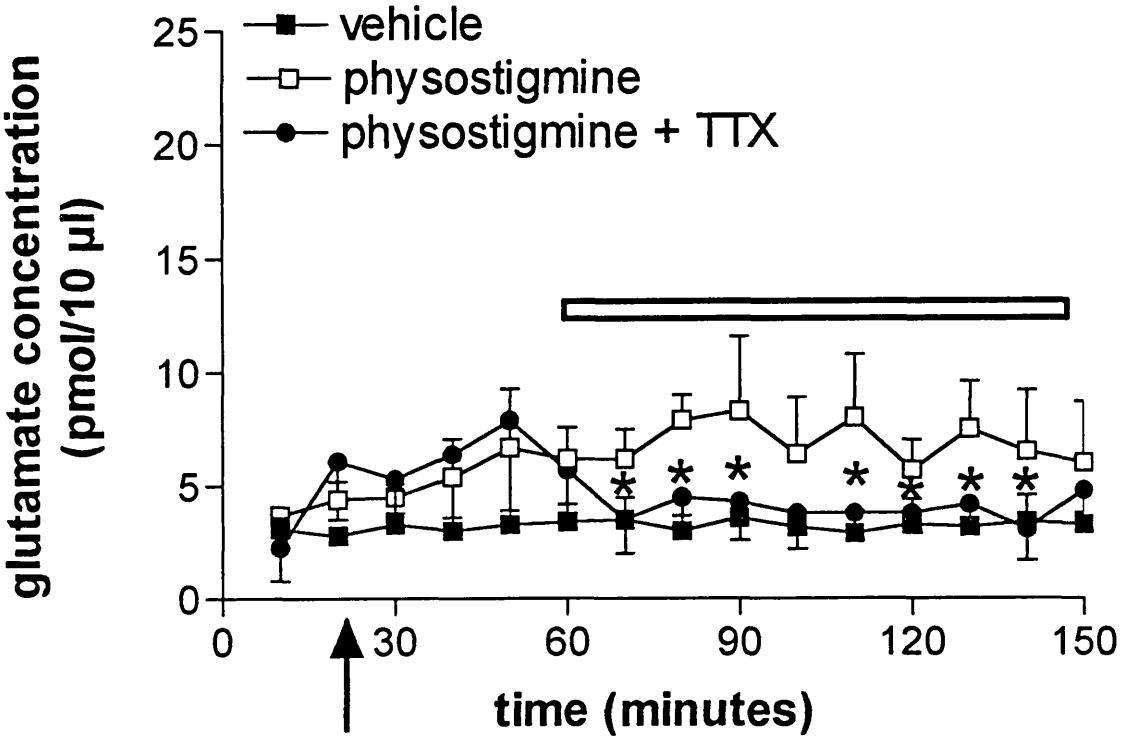
Effect of intramuscular injection of physostigmine (0.3 mg/kg at the beginning of the second collection period, arrow, n = 7) on glutamate concentration in striatal dialysate, compared to vehicle injection (mPBS, arrow, n = 7). Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared with the LSD test. * = $P < 0.05$.

Figure 5,2



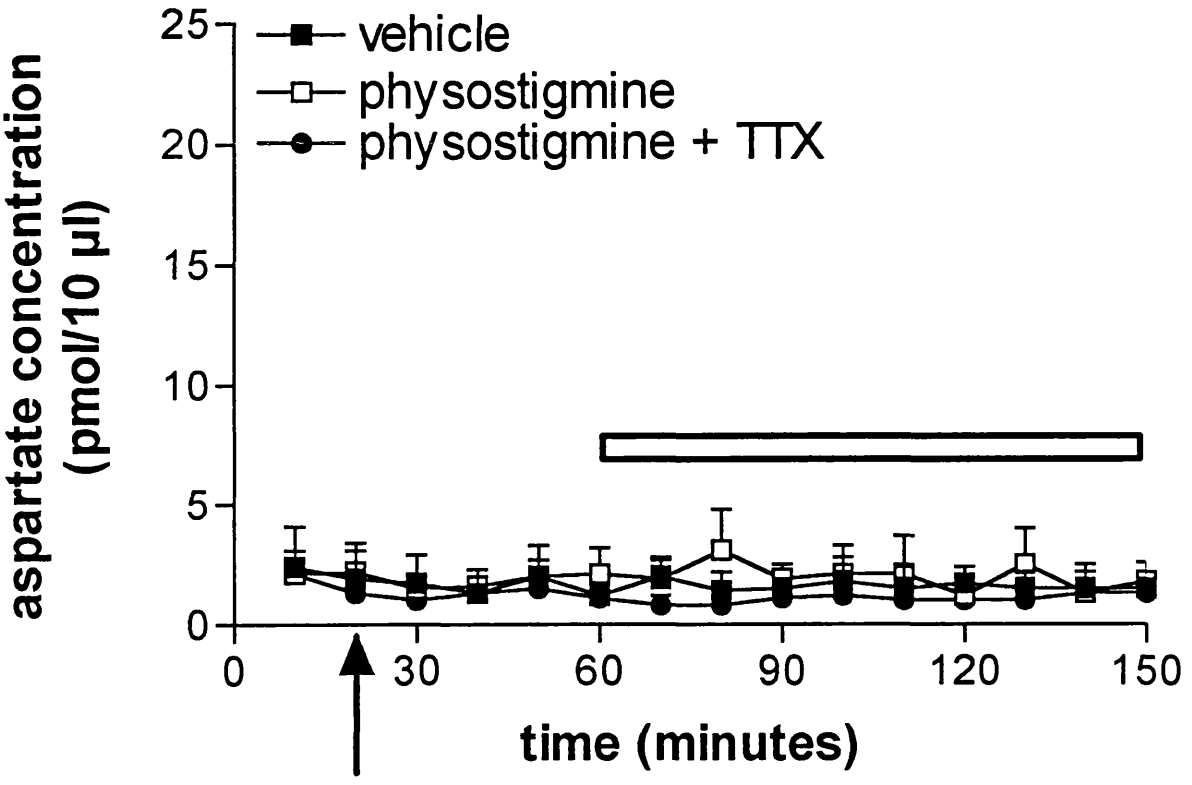
Effect of intramuscular injection of physostigmine (0.3 mg/kg, at the beginning of the second collection period, arrow, n = 7) on aspartate concentration in striatal dialysate, compared to vehicle injection (mPBS, arrow, n = 7). No significant effect was detected. Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared with the LSD test.

Fig 5,3



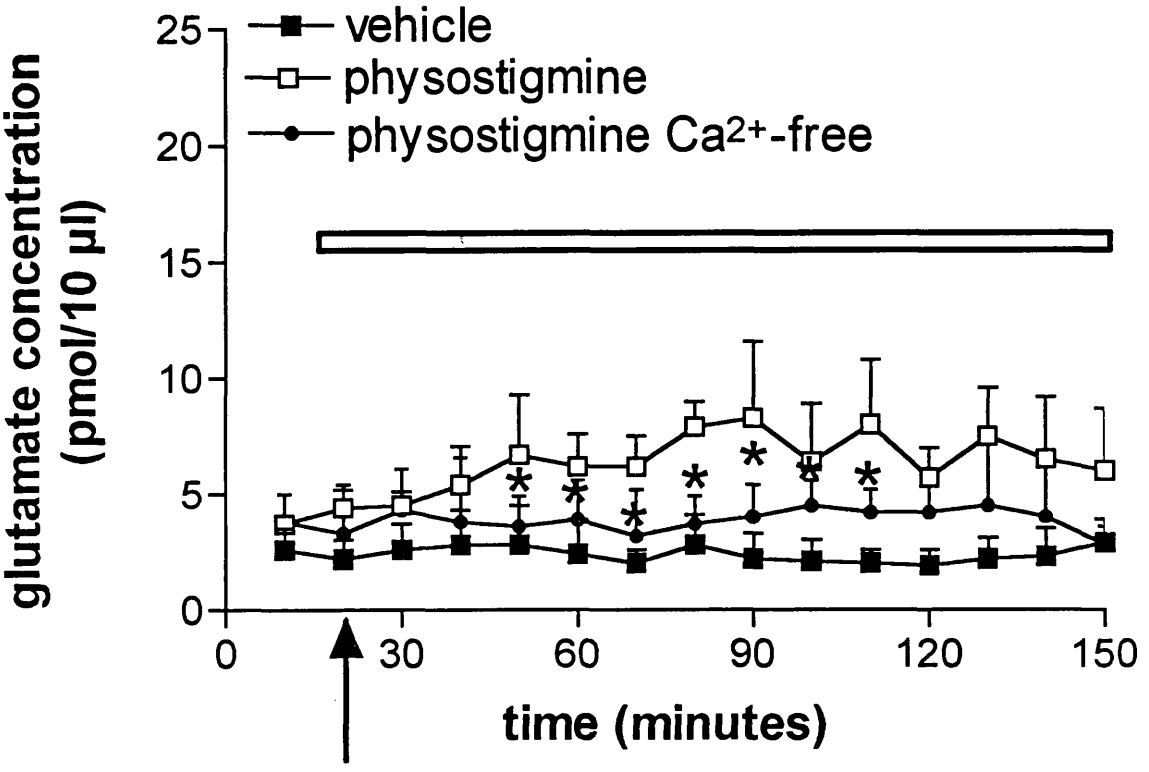
Tetrodotoxin sensitivity of the physostigmine-induced increase in glutamate concentration in striatal dialysate (bar = tetrodotoxin, topically applied to the frontal cortex from sample 6-15, 10 μM, n = 6; arrow: intramuscular physostigmine injection). Data were analysed using an unbalanced repeated measures ANOVA followed by the Student's t-test. * = $P < 0.05$.

Fig 5,4



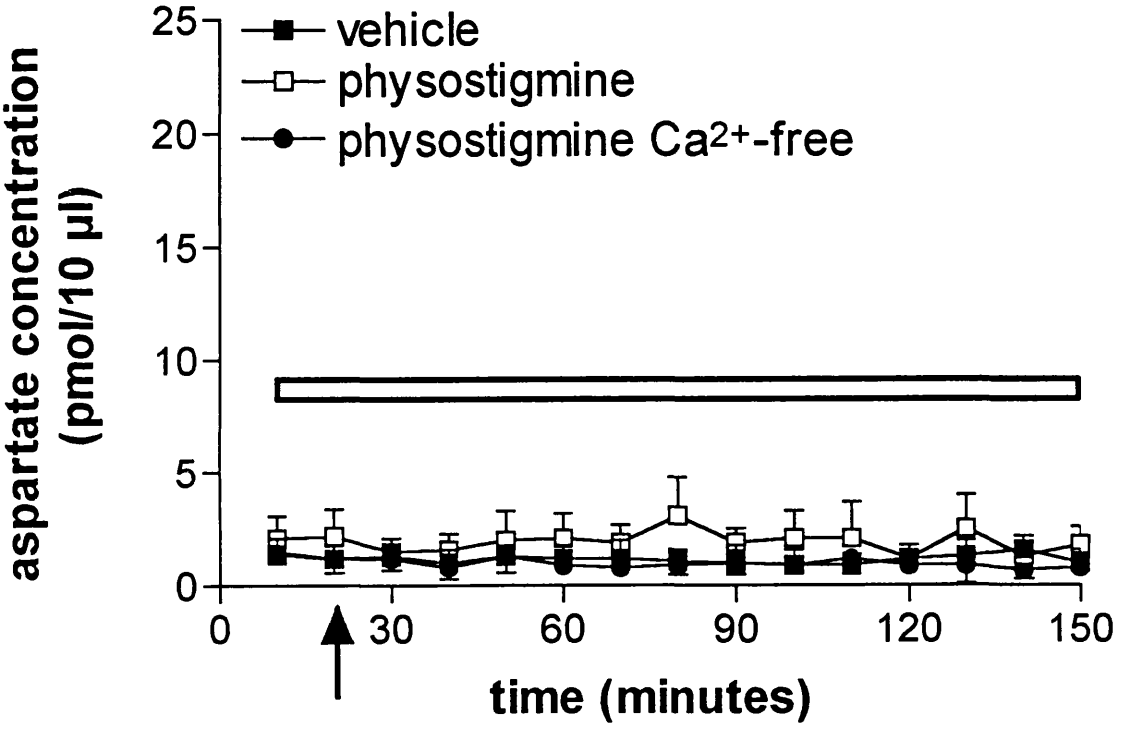
Comparison of aspartate concentration in striatal dialysate when physostigmine is injected (0.3 mg/kg intramuscularly at the beginning of the second collection period, n = 6) and injection intramuscularly combined with topical application to the frontal cortex of tetrodotoxin (bar, n = 6). Data were analysed using an unbalanced repeated measures ANOVA followed by the Student's t-test. No significant differences were detected.

Fig 5,5



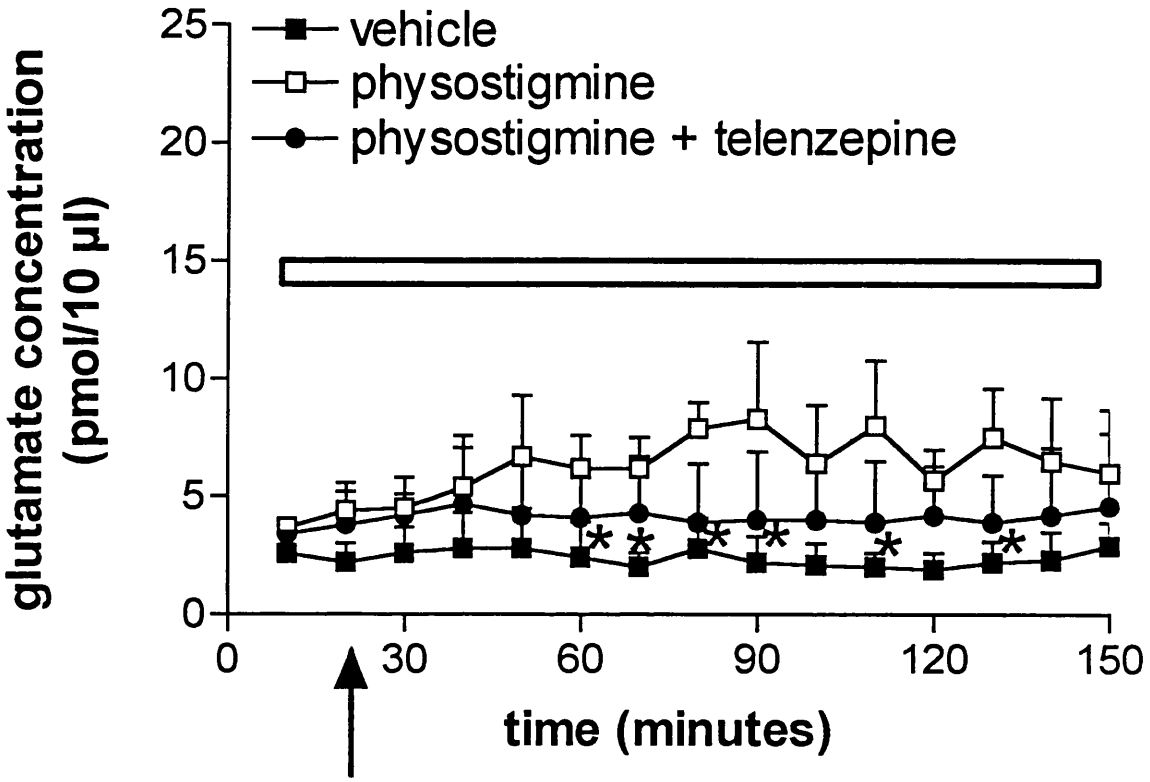
Ca^{2+}-dependency (perfusion of microdialysis probe with Ca^{2+} -free perfusion fluid from moment of implantation, n = 7) of physostigmine-induced (0.3 mg/kg intramuscularly at the beginning of the second collection period, n = 6) increase in glutamate concentration in striatal dialysate. Data were analysed using an unbalanced repeated measures ANOVA followed by the Student's t-test. * = $P < 0.05$.

Fig 5,6



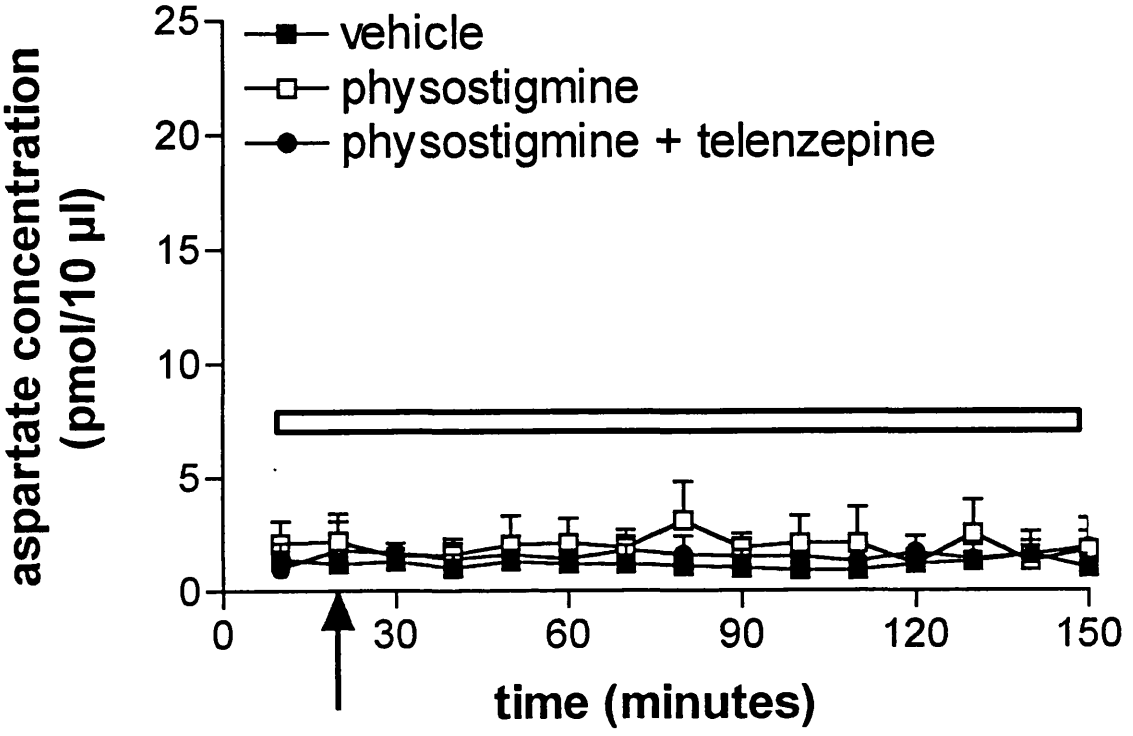
Comparison of aspartate concentration in striatal dialysate when physostigmine is injected (0.3 mg/kg intramuscularly at the beginning of the second collection period, n = 6) and injection of physostigmine i.m. combined with microdialysis with Ca^{2+}-free medium (from moment of implantation, n = 7). Data were analysed using an unbalanced repeated measures ANOVA followed by the Student's t-test. No significant differences were detected.

Fig 5,7



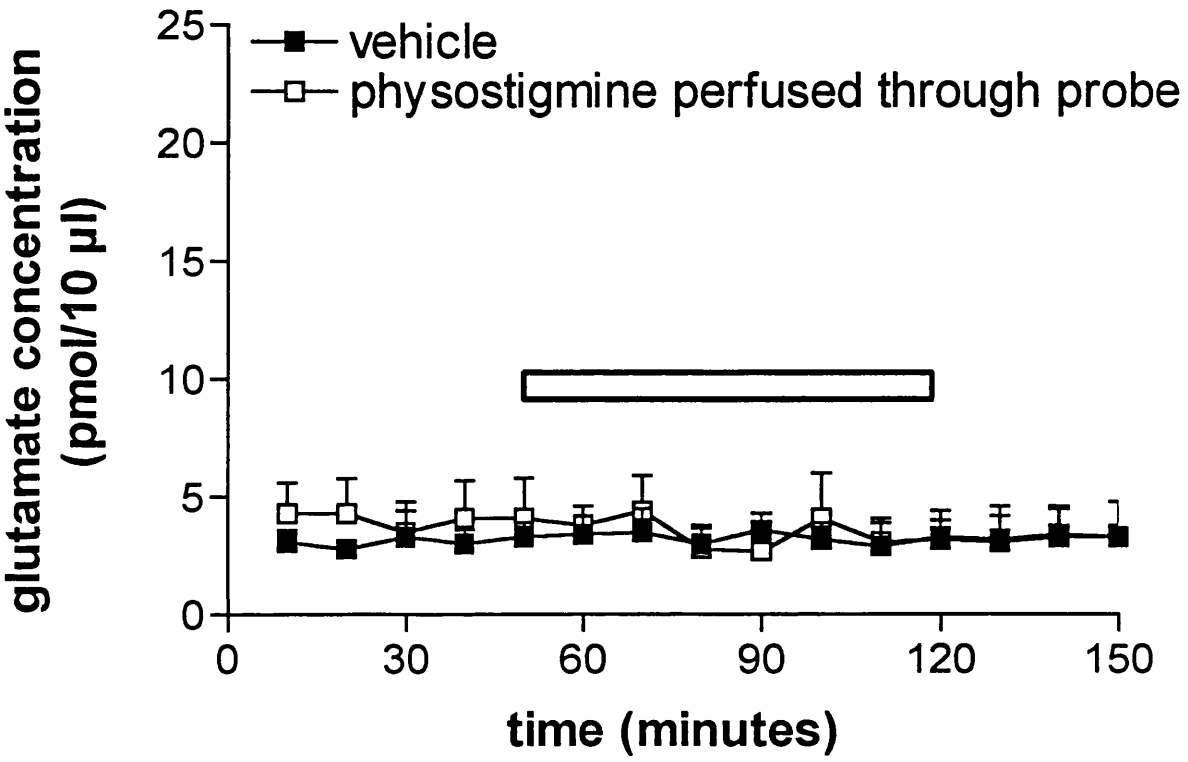
Effect of topical application to the frontal cortex of the M_1 antagonist telenzepine (applied at the beginning of all collection periods = bar, 5 μM, n = 6) on physostigmine-induced (0.3 mg/kg intramuscularly at the beginning of the second collection period, n = 7) increase in glutamate concentration in striatal dialysate. Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared by oneway ANOVA followed by the LSD test. * = $P < 0.05$.

Fig 5,8



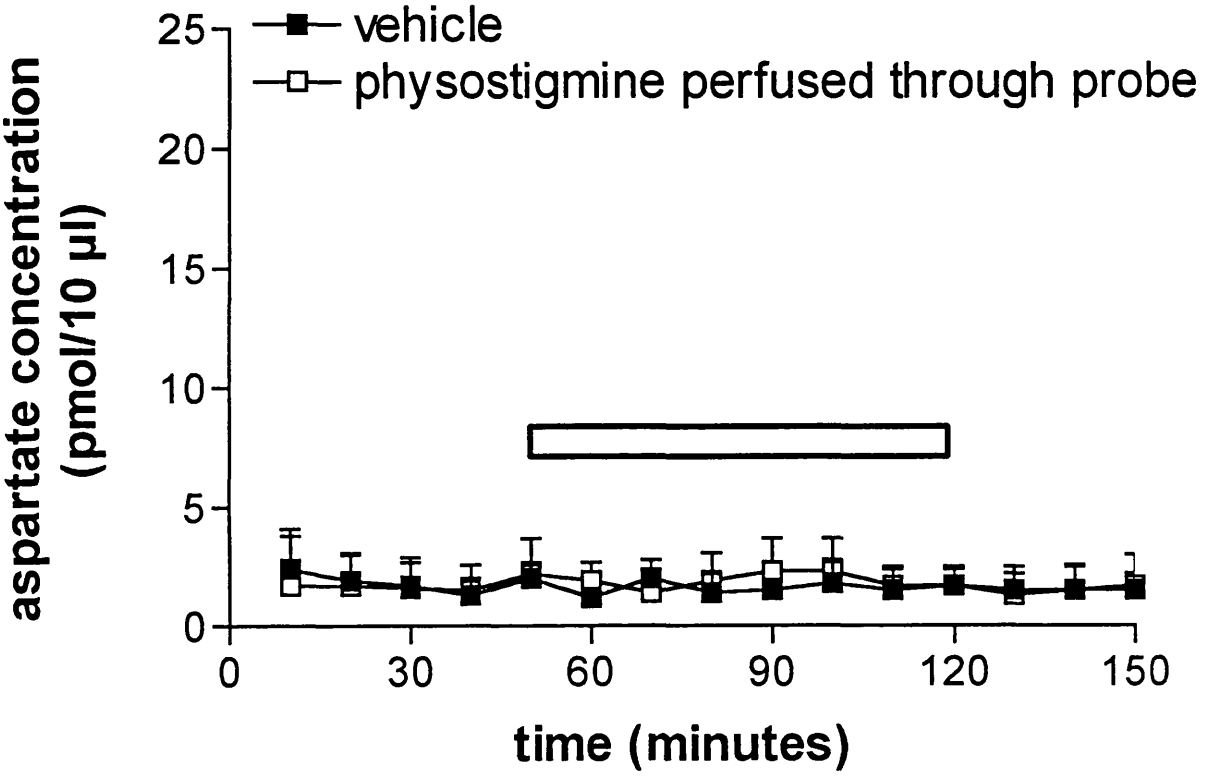
Comparison of aspartate concentration in striatal dialysate when physostigmine is injected (0.3 mg/kg intramuscularly at the beginning of the second collection period, n = 6) and injection of physostigmine intramuscularly, combined with topical application of telenzepine (beginning of each collection period = bar, 5 μM, n = 6). Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared by oneway ANOVA followed by the LSD-test. No significant differences were detected .

Fig 5,9



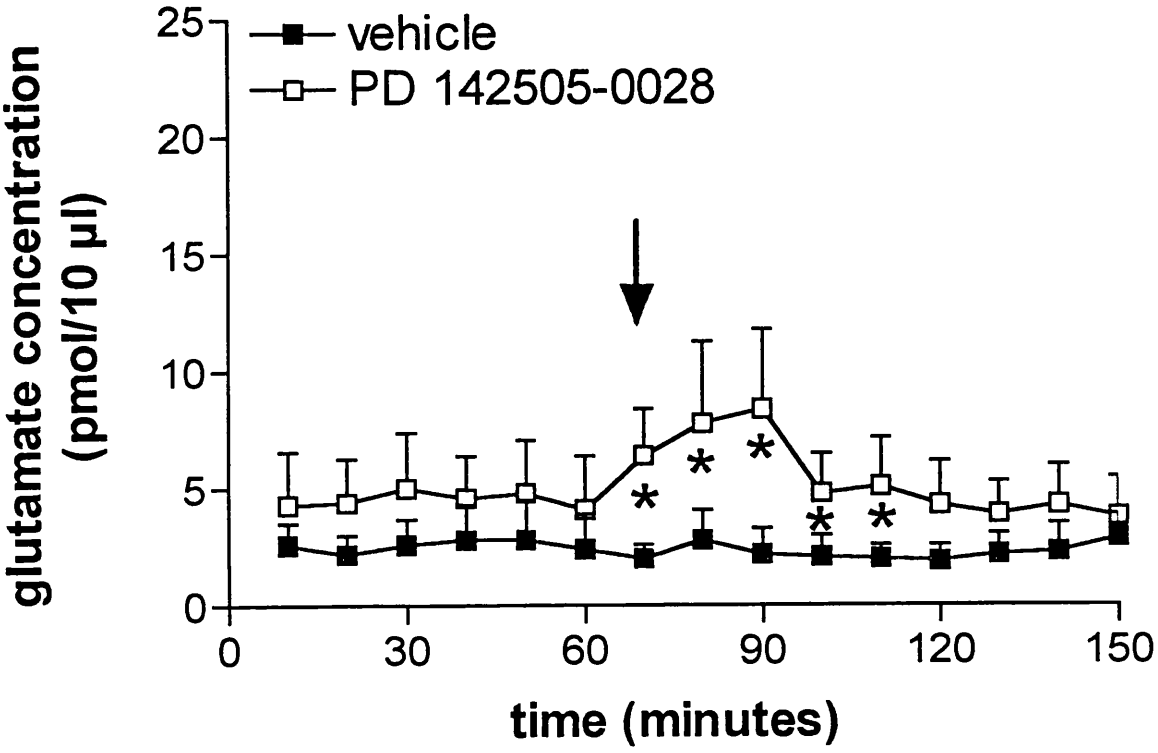
Comparison of the effect on glutamate concentration in striatal dialysate when dialysis without (mPBS, n = 10) or with physostigmine included in the dialysis fluid (bar, 10 μM, n = 6). Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared using oneway ANOVA followed by the LSD-test. No significant differences were detected.

Fig 5,10



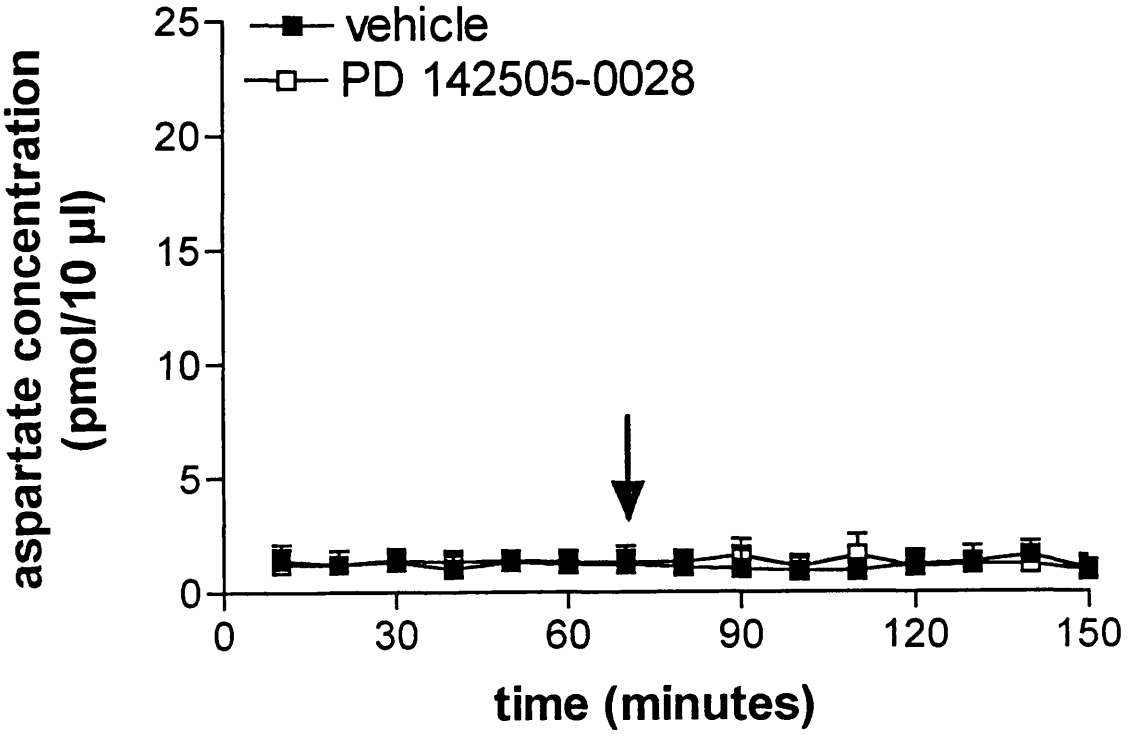
Comparison of the effect on aspartate concentration in striatal dialysate when dialysis without (mPBS, n = 10) or with physostigmine included in dialysis fluid (bar, 10 μM, n = 6). Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared using oneway ANOVA followed by the LSD-test. No significant differences were detected.

Fig 5,11



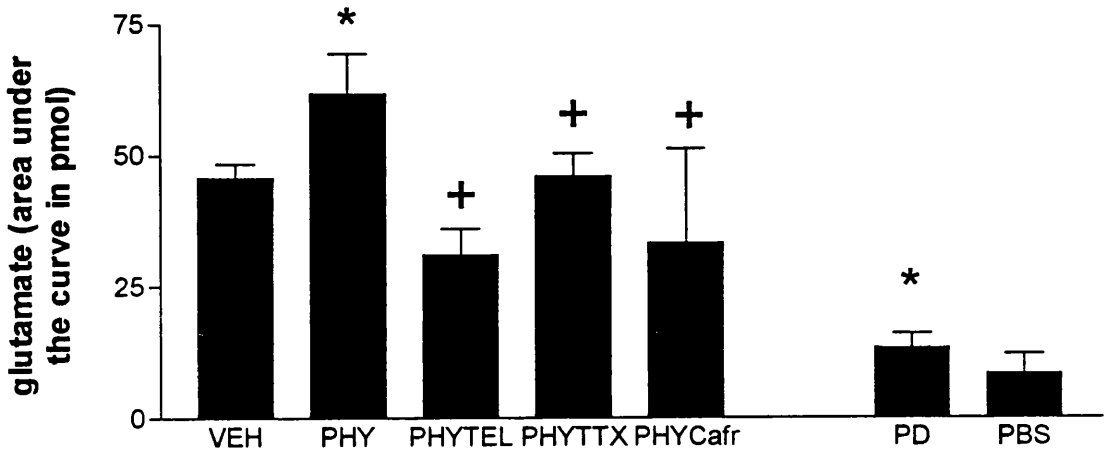
Effect of the partial M_1 agonist PD 142505-0028, topically applied to the frontal cortex (arrow, 10 μM, n = 6) compared to topical application of vehicle (arrow, n = 10) on glutamate concentration in striatal dialysate. Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared using oneway ANOVA followed by the Student's t-test. * = $P < 0.05$.

Fig 5,12



Comparison of effect of the partial M_1 agonist PD 142505-0028 (arrow, 10 μM, n = 6) and topical application of vehicle (arrow, n = 10) on aspartate concentration in striatal dialysate. Data were analysed using an unbalanced repeated measures ANOVA. Individual means were compared using oneway ANOVA followed by the Student's t-test. No significant differences were detected in any sample.

Fig 5,13



Effect of topical application of drugs to the frontal cortex on GLU concentrations in striatal dialysate, expressed as area under the curve (pmol). Data were analysed using a Kruskal-Wallis ANOVA followed by either a Mann Whitney U test or a LSD test, as appropriate. * = $P < 0.05$ as compared to the vehicle effect. + = $P < 0.05$ as compared to the physostigmine effect. (VEH = vehicle = PBS; PHY= physostigmine 0.3 mg/kg intramuscular injection; PHYTEL = physostigmine 0,3 mg/kg intramuscular injection + topical application of telenzepine (5μM); PHYTTX = physostigmine 0.3 mg/kg intramuscular injection + topical application of tetrodotoxin 10 μM; PHYCafr = physostigmine injected intramuscularly, dialysis with Ca^{2+}-free fluid; PD = PD 142505-0028 10 μm).

5.5 Discussion

In this Chapter the effect of peripherally administering physostigmine and topical application of a novel selective M_1 partial agonist on the activity of pyramidal neurones in layer V of the rat cortex were investigated. An increase in glutamate but not aspartate concentration in the striatum after peripherally administered, but not after local application of physostigmine through the dialysis probe was observed. In addition, topical application of an M_1 agonist to the frontal cortex increased glutamate but not aspartate concentration in the striatum. That the compound PD 142505-0028 is a partial muscarinic 1 agonist is suggested by binding studies in membranes from transfected cells (Jaen et al., 1995) For example the compound binds with high affinity to [^{3}H] labelled muscarinic receptors on rat cortical membranes (IC_{50}: 28 nM), it displays a binding shift indicative of significant muscarinic agonist activity, and increases PI turnover (51% of carbachol's maximal effect) in m1-transfected cells at concentrations that produce no significant stimulation of m3- or m5-transfected cells.

The change in glutamate following cortical stimulation is consistent with the tenet that this amino acid is the neurotransmitter of the corticostriatal pathway (Fonnum et al., 1984). The most likely explanation for the increase in glutamate concentrations in the striatum after peripheral administration of physostigmine is that an increased concentration of ACh in the cortex facilitates neurotransmission in the corticostriatal pathway, resulting from an increased likelihood that a given cortical pyramidal neurone is depolarised by endogenous EAA's. Although the receptor(s) which mediate this effect need to be characterised in more detail the telenzepine experiment suggests that the effect of physostigmine on striatal outflow is mediated by activation of muscarinic (probably M_1) receptors on corticostriatal neurones. This conclusion is strengthened by the observation that PD142505-0028 had an effect not dissimilar from physostigmine. Furthermore, other studies have shown that M_1 receptor activation produces a prolonged facilitation of cortical neurones (McCormick & Prince, 1985), rendering them more likely to depolarise following

application of glutamatergic agonists.

The results of this study suggest that administration of an anticholinesterase or cholinergic agonist would increase the (reduced) excitability of cortical pyramidal neurones in AD and may therefore ameliorate the effects of glutamatergic hypoactivity on cognitive function (Francis et al., 1993). It remains to be investigated whether stimulation of both ion gated (nicotine) and PKC-linked (M_1) receptors gives a similar increase in excitability as an acetylcholinesterase inhibitor.

Well designed trials with adequate numbers of subjects showed that the anticholinesterase Tacrine benefits some patients, expressed either as improvement in the core deficits of AD, or as a reduced rate of deterioration (Byrne & Arie, 1994). However, only few patients benefit greatly. While it is clear that Tacrine is not an ideal cholinesterase inhibitor, another approach to supplement the deficit in ACh in the AD brain, could be to use M_1 agonists. The results from this study suggest that in terms of increasing the excitability of cortical pyramidal neurones a selective M_1 agonist could be at least as useful as any improved cholinesterase inhibitor. However, the functional status of the M_1 receptor in AD needs to be clarified (Pearce & Potter, 1991; Flynn et al., 1995). In view of this it may transpire that the most effective treatment requires polypharmacy.

Chapter 6

Evidence that the increase in glutamate and aspartate concentration in the striatum, induced by an M_1 agonist, is potentiated by the 5-HT_{1A} antagonist WAY100135

6.1 Introduction

In Chapter 4 and 5 it was observed that both a selective M_1 agonist and a selective 5-HT_{1A} antagonist increase activity of cortical pyramidal neurones *in vivo*. In this Chapter it was investigated whether these drugs would be able to potentiate each others action.

6.2 Materials and Methods

The following series of experiments were performed:

Drugs applied to the frontal cortex :

A) vehicle (mPBS; n=8)

B) PD 142505-0028 (10 μM; n=5)

C) WAY 100135 (50 μM; n=10)

D) PD 142505-0028 (10 μM) coapplied with WAY 100135 50 μM (n=7)

E) PD 142505-0028 coapplied with tetrodotoxin 10 μM (n=5)

F) PD 142505-0028, dialysis with Ca^{2+}-free medium (n=5)

G) WAY 100135 coapplied with tetrodotoxin (10 μM; n=6)

H) WAY 100135, dialysis with Ca^{2+}-free medium (n=6)

I) WAY 100135 coapplied with PD 142505-0028 and tetrodotoxin (n=5)

J) WAY 100135 coapplied with PD 142505-0028, dialysis with Ca^{2+}-free medium (n=6)

6.3 Results

6.3.1 Glutamate

A significant group difference was noted when samples 1-6 were compared ($F = 5.1$; $df = 2,19$; $P < 0.05$). For this reason data in this chapter are presented as increases from baseline.

PD 142505-0028, topically applied at the beginning of the ninth sample significantly ($P < 0.05$, Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test) increased glutamate concentration in striatal dialysate in sample 11, (89 ± 31 % versus 264 ± 85 %, $P < 0.05$, see Fig. 6,1).

Coapplied tetrodotoxin reduced the effect in the eleventh sample significantly (reduction from 264 ± 85 % to 108 ± 12 %, $P < 0.05$, see Fig. 6,3). Omitting Ca^{2+} from the perfusion fluid reduced the effect significantly (from 264 ± 85 % to 117 ± 32 %, $P < 0.05$, see Fig. 6,5).

Significant differences between the effect induced by PD 142505-0028 and the effect by the combination of PD 142505-0028 and WAY 100135 were observed in sample 11 (potentiation of 264 ± 85 % to 582 ± 164 %, $P < 0.05$, see Fig. 6,7).

Coapplied tetrodotoxin reduced the increase induced by the combination of drugs in sample 11 (reduction from 581 ± 164 % to 179 ± 94 %, $P < 0.05$, see Fig. 6,9).

Omitting Ca^{2+} from the dialysis fluid reduced the effect of the combination of drugs significantly in sample 11 (reduction from 581 ± 164 % to 169 ± 53 %, $P < 0.05$, see Fig. 6,11).

The effect of the WAY 100135-induced increase in glutamate concentration in striatal dialysate was tetrodotoxin sensitive (reduction from 149 ± 61 % to 101 ± 25 %, $P < 0.05$, see

Fig. 6,13).

Ca^{2+}-dependency of the WAY 100135-induced increase in glutamate concentration in striatal dialysate was observed in sample 11 (reduction from 149 ± 61 % to 74 ± 17 %, $P < 0.05$, see Fig 15).

6.3.2 Aspartate.

Only the combined application of PD 142505-0028 together with WAY 100135 caused a significant ($P < 0.05$, Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test) increase in aspartate concentration in striatal dialysate. PD 142505-0028 versus PD 142505-0028 combined with WAY 100135, sample 10, (101 ± 21 % versus 222 ± 146 %, $P < 0.05$), sample 11, (82 ± 16 % versus 323 ± 99 %, $P < 0.05$), sample 14, (74 ± 22 versus 98 ± 20, $P < 0.05$, see Fig. 6.8).

Coapplying tetrodotoxin with the combination of drugs significantly reduced the increase in aspartate concentration in dialysate in sample 10 (from 222 ± 146 to 85 ± 10 %, $P < 0.01$) and in sample 11 (from 323 ± 99 to 108 ± 40 %, $P < 0.01$, see Fig. 6,10).

Omitting Ca^{2+} from the dialysis fluid reduced the increase in sample 10 (from 222 ± 146 to 93 ± 16 %, $P < 0.01$) and in sample 11 (from 323 ± 99 to 79 ± 15 %, $P < 0.01$, see Fig. 6,12).

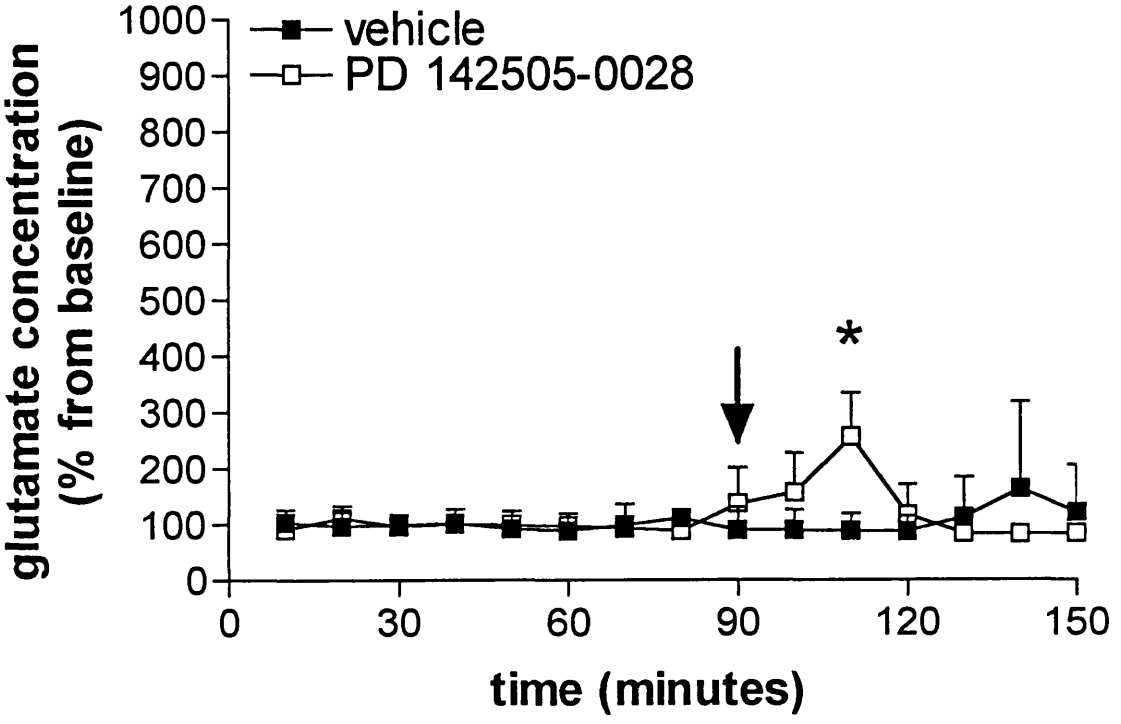
(Figures 6,2; 6,4; 6,6; 6,12 and 6,14 are given for illustration only).

6.3.3 Aspartate and Glutamate area under the curve and ASP/GLU ratio.

Treatment	area under curve ASP	area under curve GLU	ratio ASP/GLU
PBS	199	285	0.7
PD	258	552	0.5
PD +TTX	302	305	1.0

PD Ca^{2+}-free	275	309	0.9
WAY	224	226	1.0
WAY +TTX	232	297	0.8
WAY Ca^{2+}-free	286	266	1.1
PD + WAY	939	1240	0.75
PDWY +TTX	586	716	0.8
PDWY CA^{2+}-free	601	718	0.8

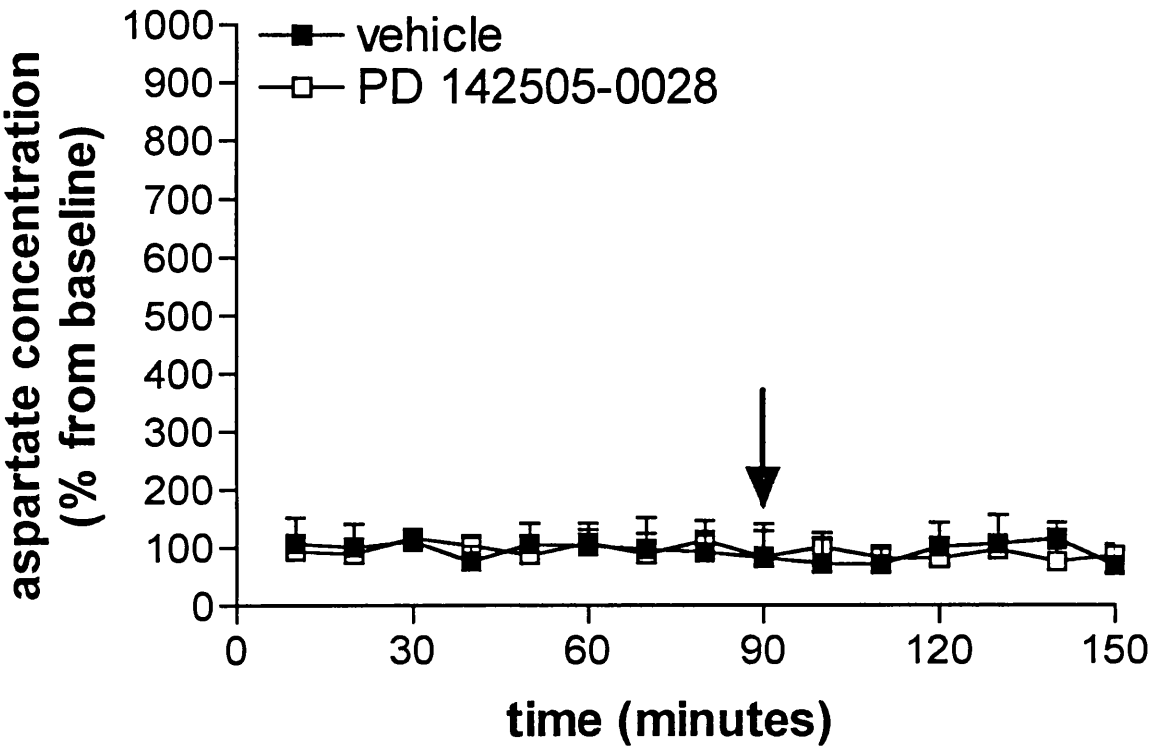
Fig 6,1



Data in Figure 6,1-6,16 are Means ± SD and are expressed as % from baseline (Mean of samples 1-6)

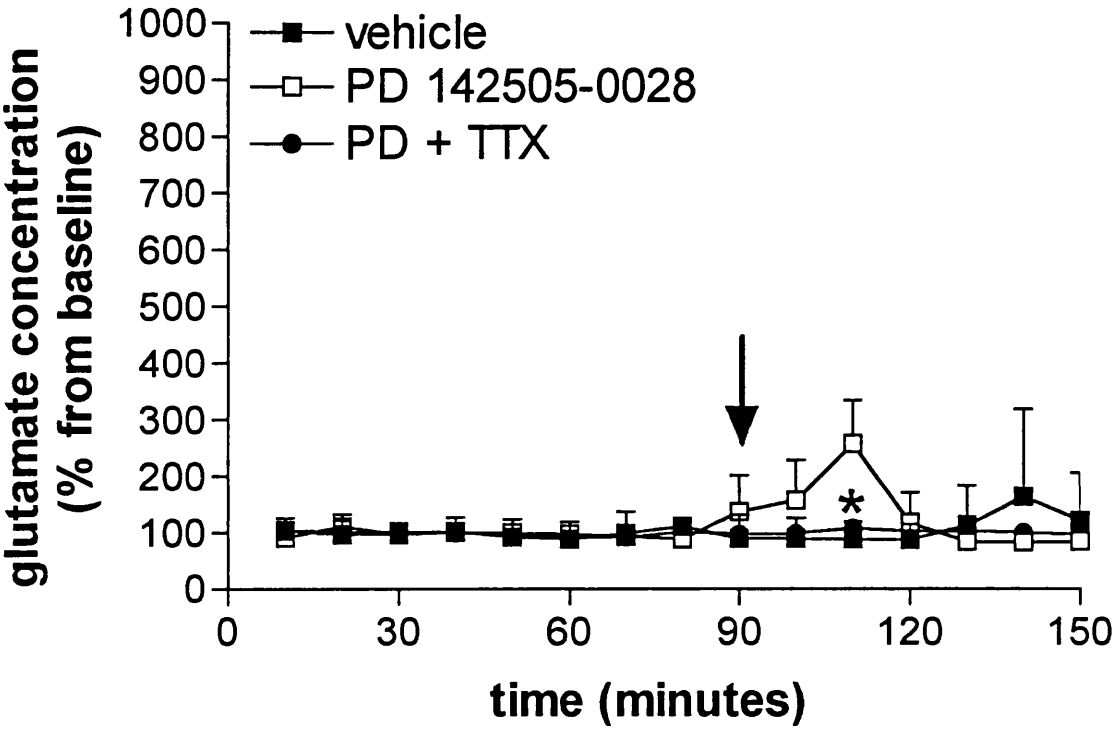
Effect of PD 142505-0028 (arrow, 10 μM, n = 5) on glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,2



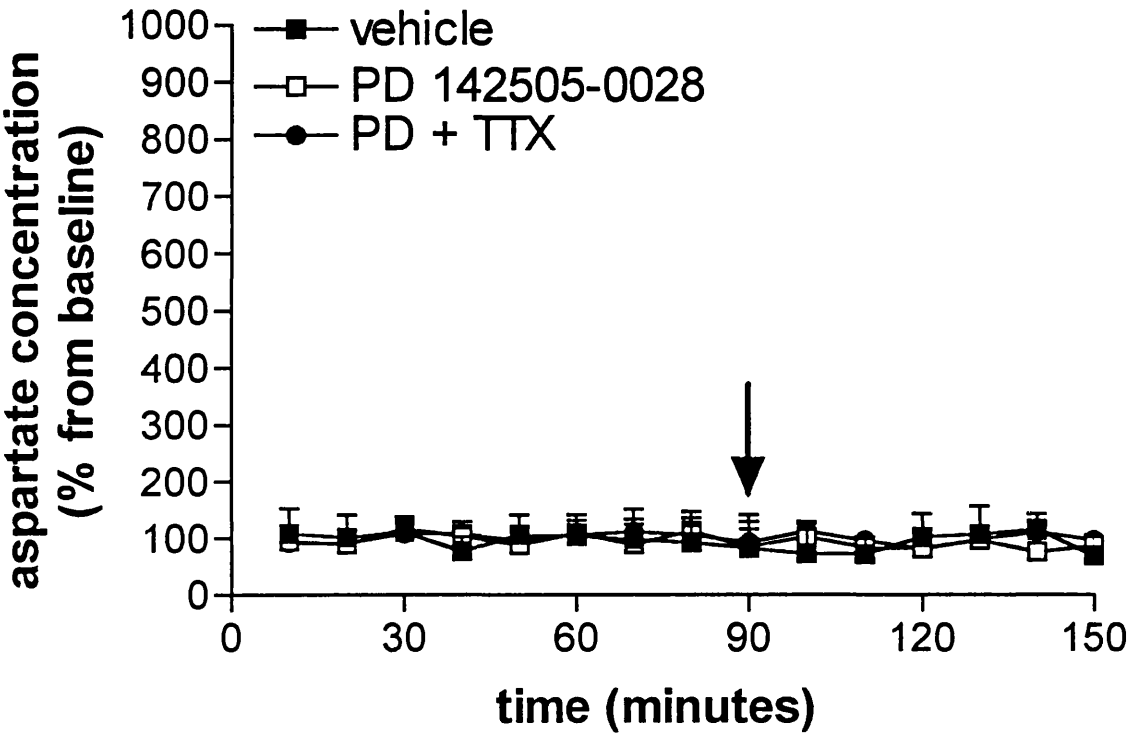
Effect of PD 142505-0028 (arrow, 10 μM, n = 5) on aspartate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. No significant effect was observed.

Fig 6,3



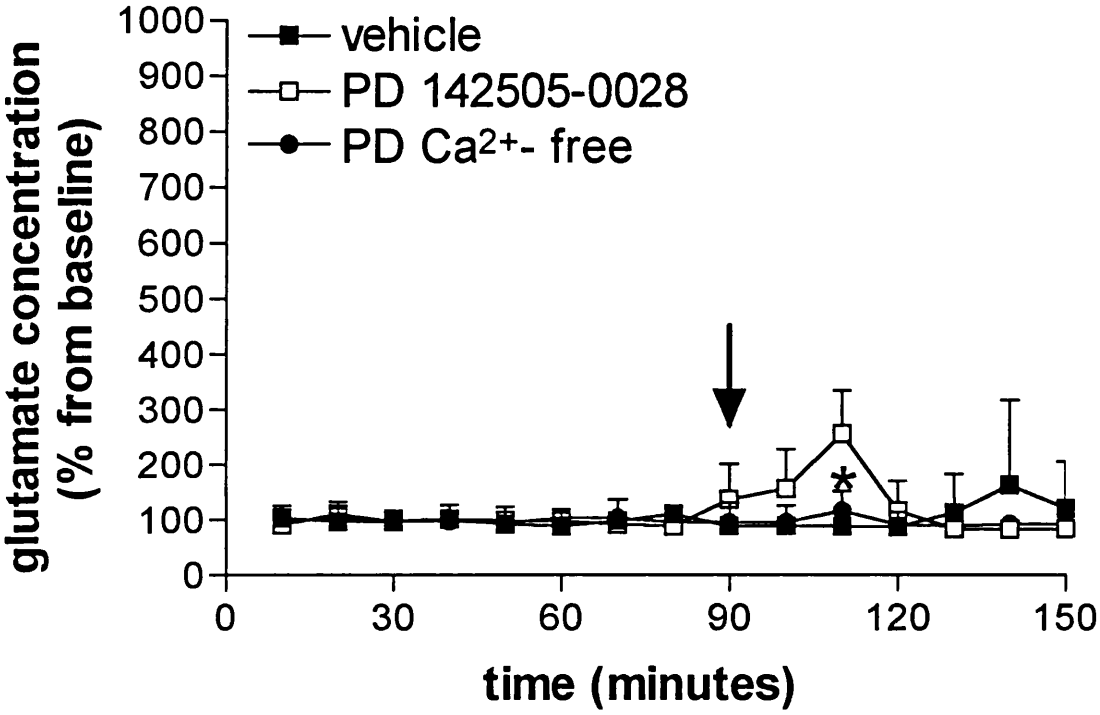
Effect of coapplied tetrodotoxin (10 μM, n = 5) on effect of PD 142505-0028 (10 μM, n = 5)crease in glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,4



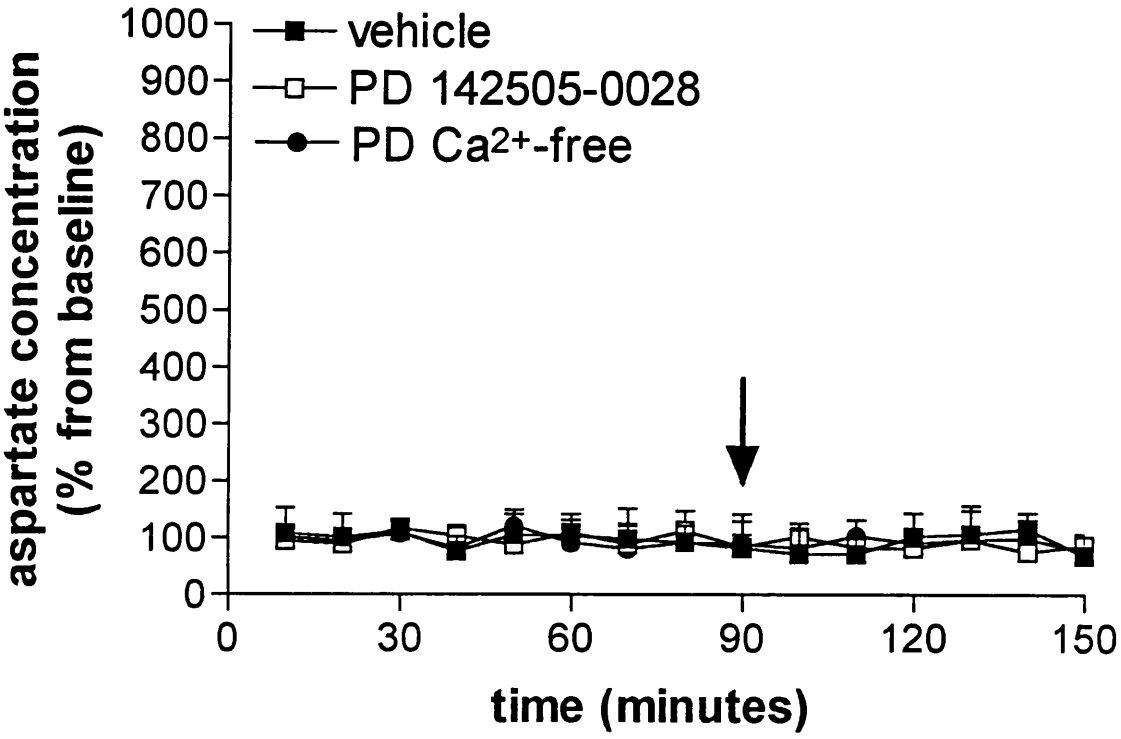
Effect of coapplied tetrodotoxin (10 μM, n = 5) on the effect of PD 142505-0028 (10 μM, n = 5) on aspartate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. No significant differences were observed..

Fig 6,5



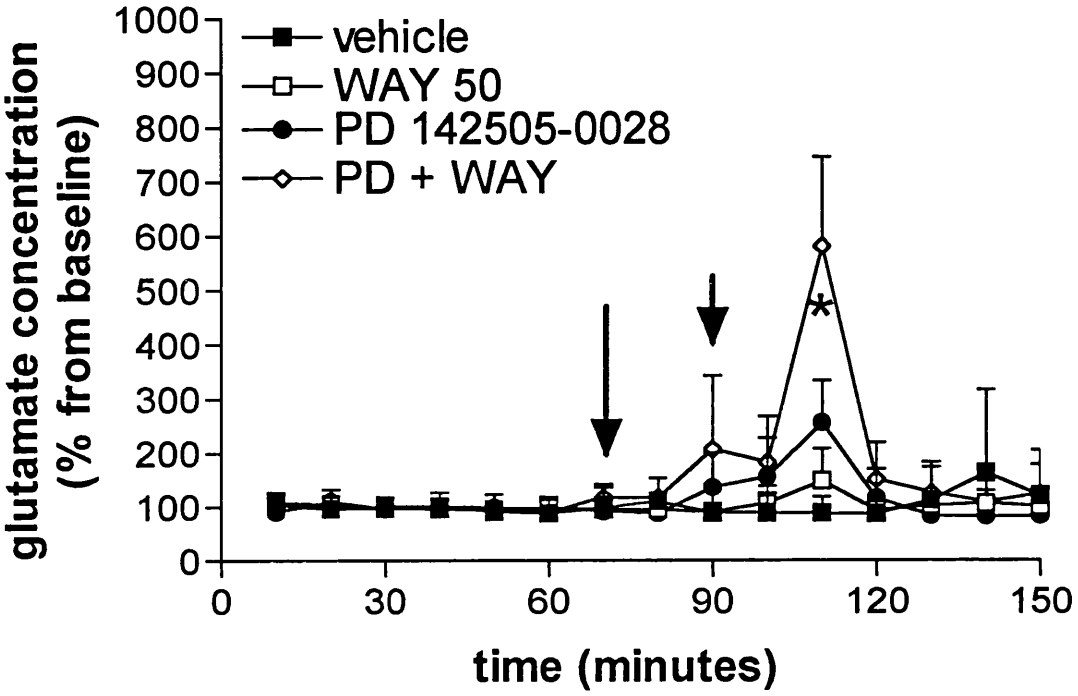
Effect of omitting Ca^{2+} from the dialysis fluid (from moment of implantation, n = 5) on PD 142505-0028 (10 μM, n = 5)-induced increase in glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,6



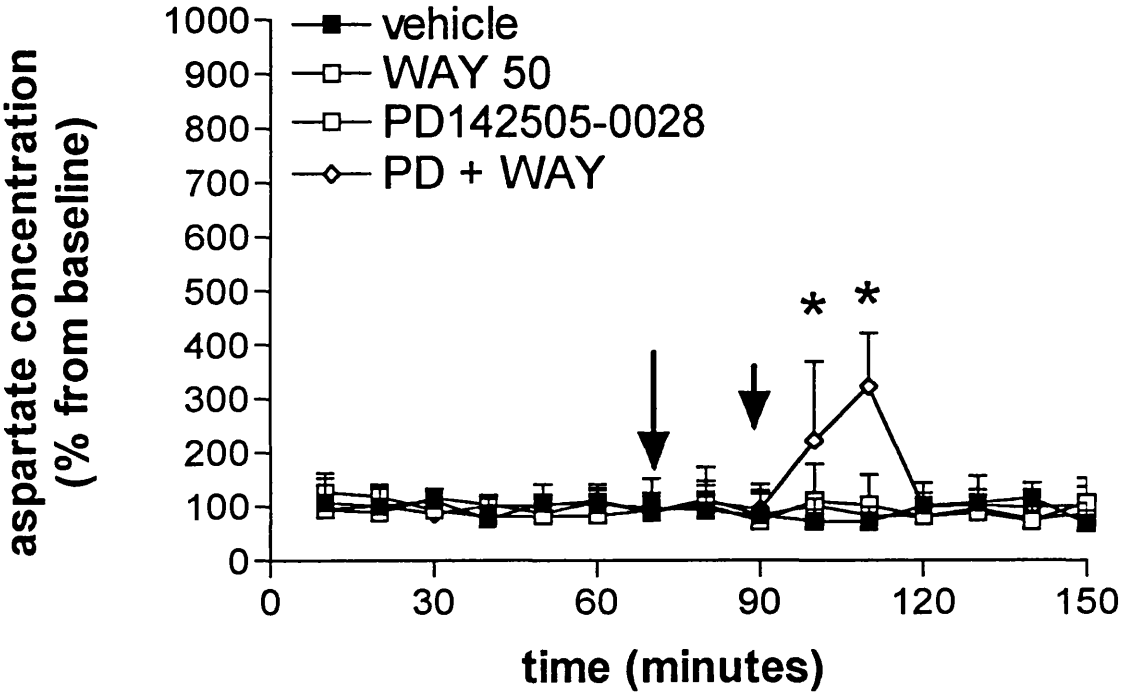
Comparison of effect of PD 142505-0028 (10 μM, n = 5) on aspartate concentration in striatal dialysate when dialysis with and without Ca^{2+} in the dialysis fluid (from moment of implantation, n = 5) . Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. No significant differences were observed.

Fig 6,7



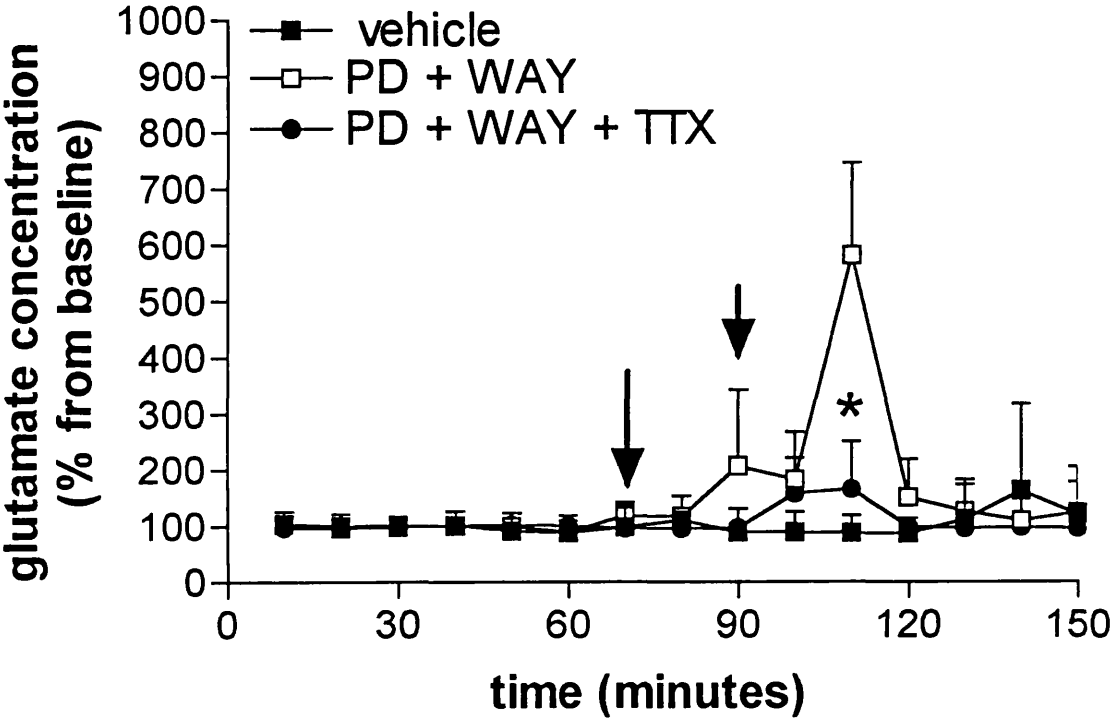
Effect of WAY 100135 (large arrow, 50 μM, n = 10), PD 142505-0028 (small arrow, 10 μM, filled, n = 5) and coapplication of a combination of the two drugs (large and small arrow, 10 and 50 μM, n = 7), on glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,8



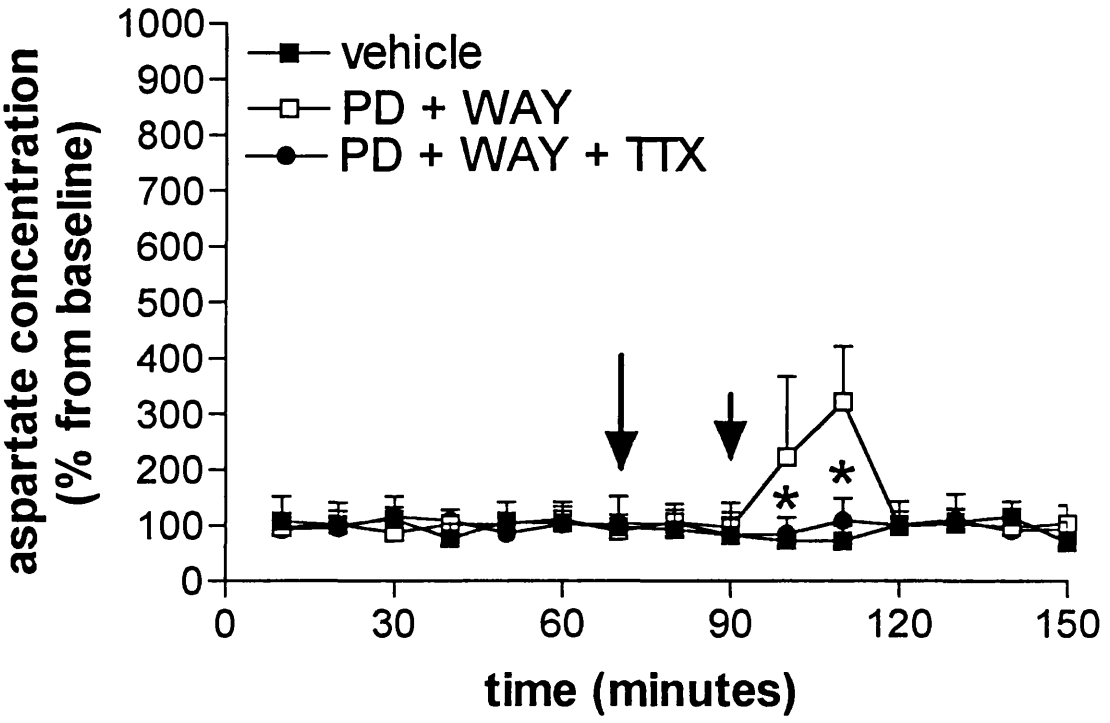
Effect of WAY 100135 (large arrow, 50 μM, n = 10), PD 142505-0028 (small arrow, 10 μM, n = 5) and coapplication of a combination of the two drugs (large and small arrow, 10 and 50 μM, n = 7), on aspartate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,9



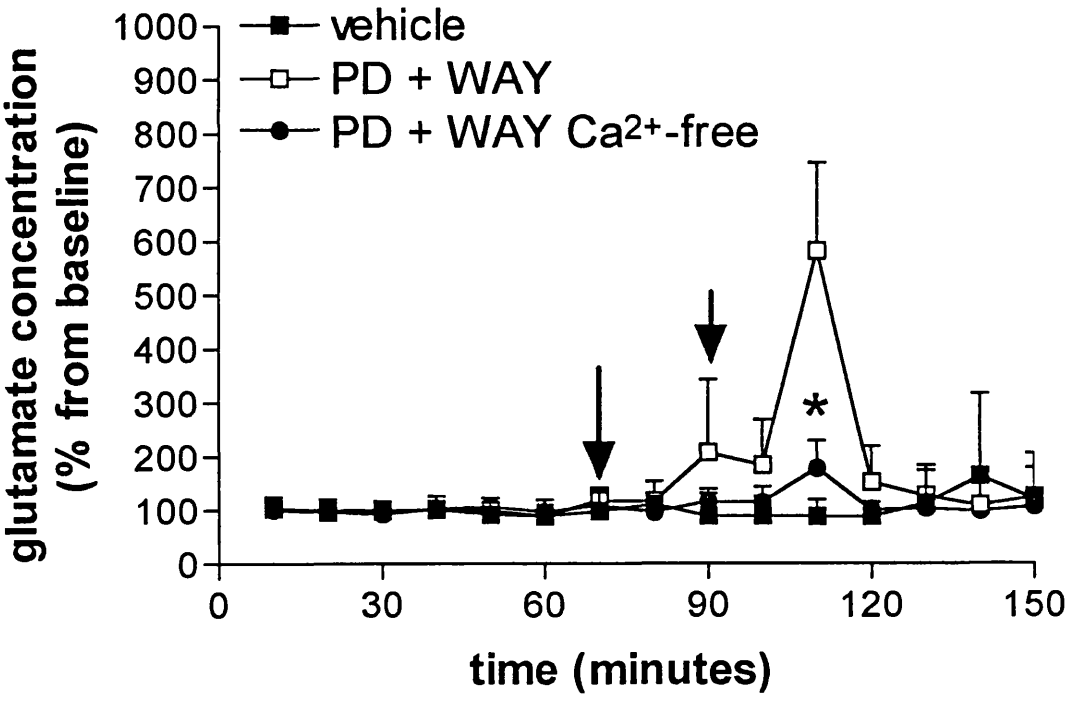
Effect of coapplied tetrodotoxin (10 μM, n = 5) on the increase in glutamate concentration in striatal dialysate, induced by a combination of WAY 100135 (50 μM) and PD 142505-0028 (10 μM), (large and small arrow, n = 7). Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,10



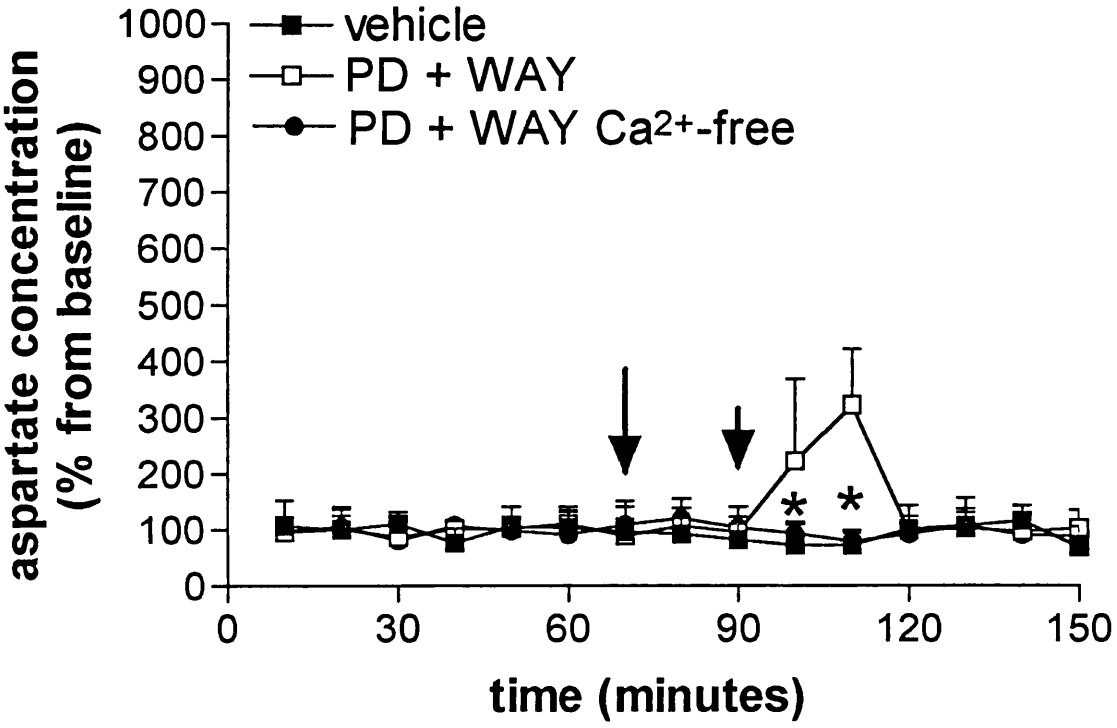
Effect of coapplied tetrodotoxin (10 μM, n = 5) on the increase in aspartate concentration in striatal dialysate, induced by a combination of WAY 100135 (50 μM) and PD 142505-0028 (10 μM), (large and small arrow, n = 7). Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,11



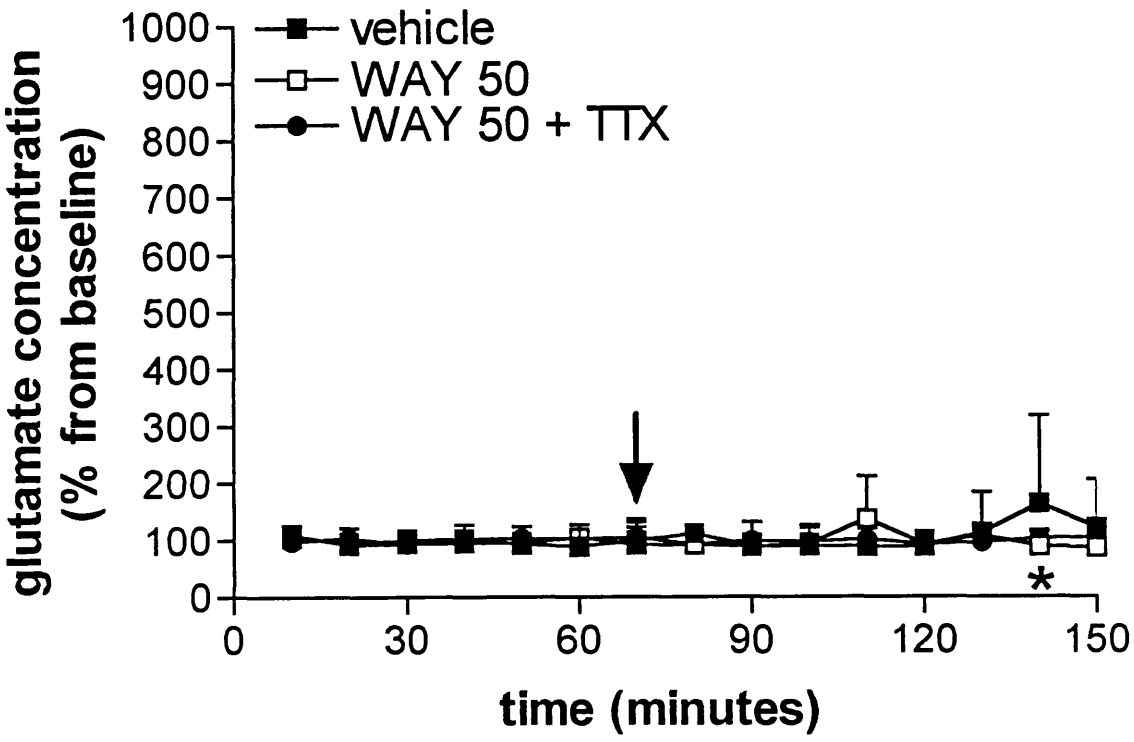
Ca^{2+}-dependency of the increase in glutamate concentration in striatal dialysate, induced by a combination of WAY 100135 (50 μM) and PD 142505-0028 (10 μM) (large and small arrow, n = 6). Ca^{2+}- free fluid perfused through the probe from moment of implantation. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,12



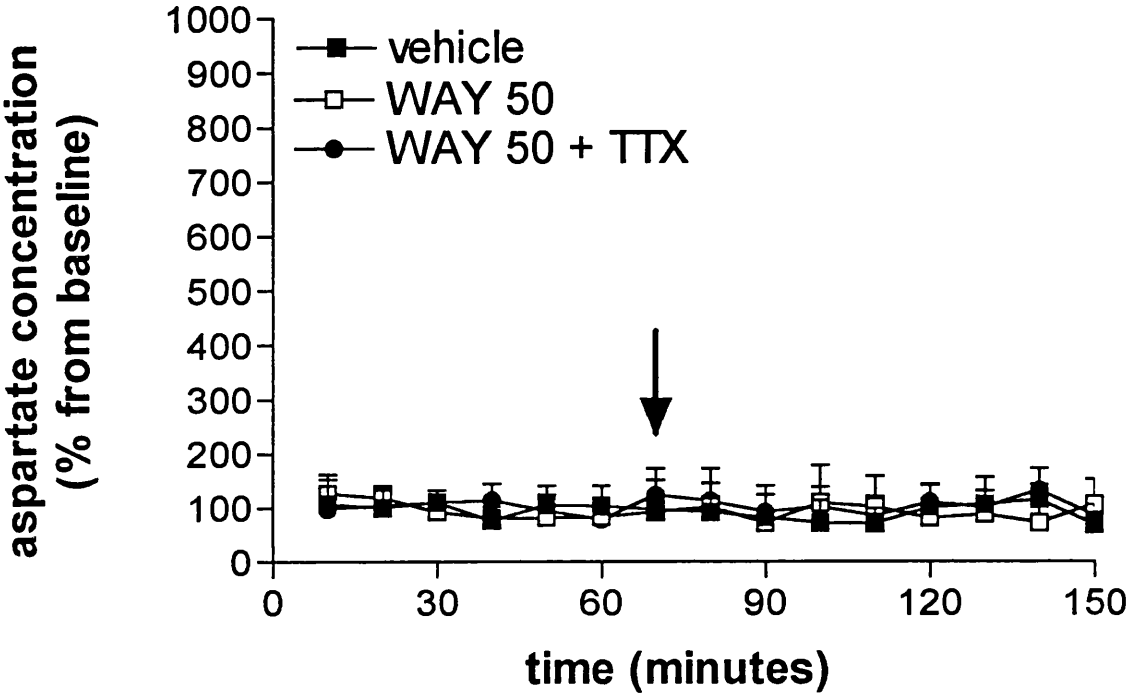
Ca^{2+}-dependency of the increase in aspartate concentration in striatal dialysate, induced by a combination of WAY 100135 (50 μM) and PD 142505-0028 (10 μM) (large and small arrow, n = 6). Ca^{2+}-free fluid perfused through the probe from moment of implantation. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,13



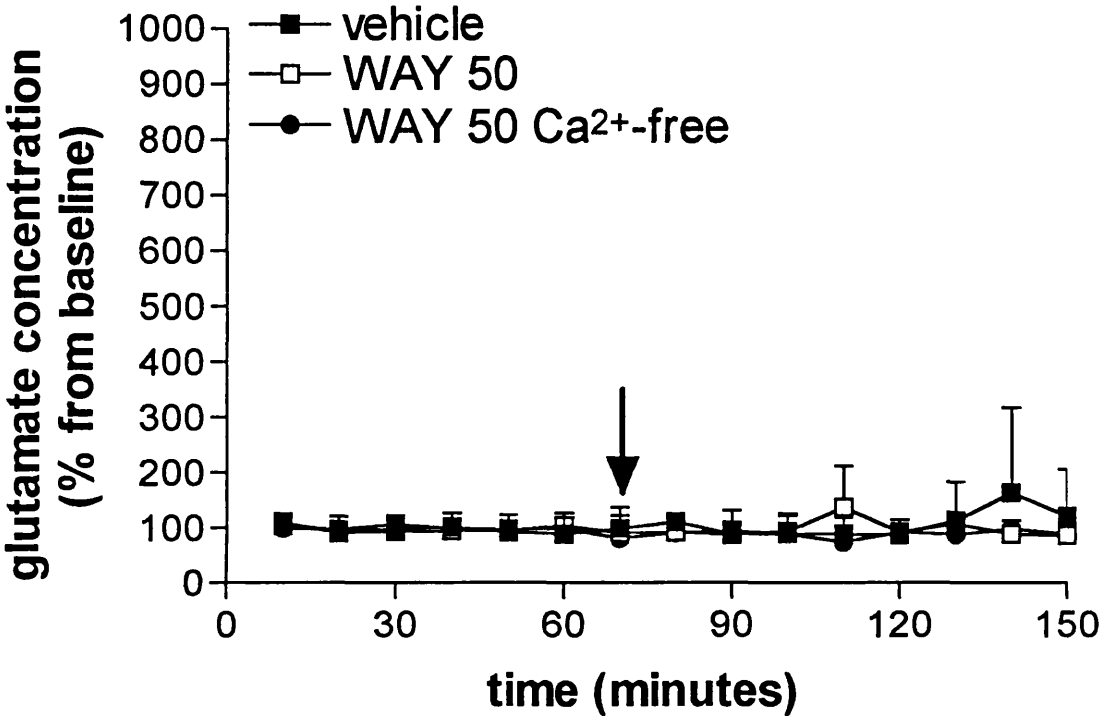
Effect of coapplied tetrodotoxin (10 μM, n = 6) on WAY 100135 (50 μM, n = 10)-induced increase in glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.

Fig 6,14



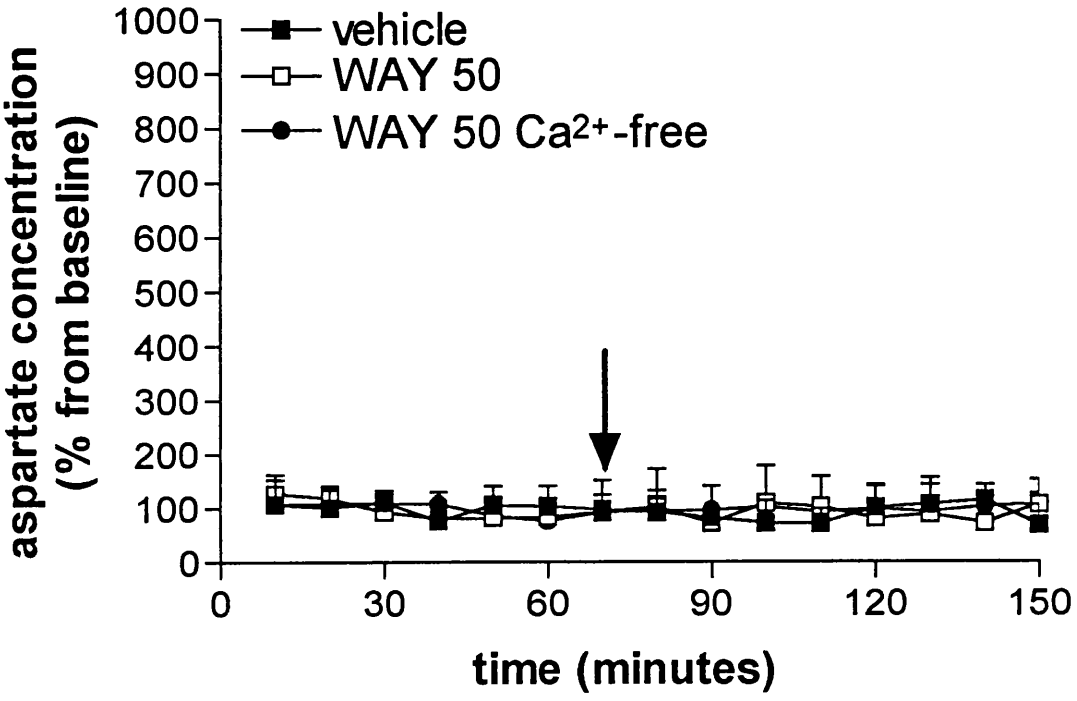
Effect of coapplied tetrodotoxin (10 μM, n = 6) on the effect of WAY 100135 M, n = 10) on aspartate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. No significant differences were observed.

Fig 6,15



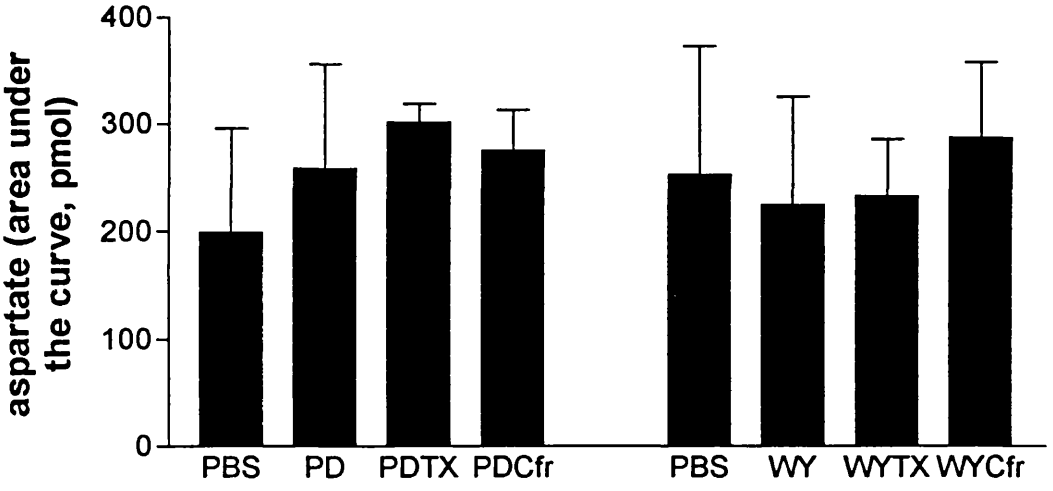
Effect of omitting Ca^{2+} from the dialysis fluid (from moment of implantation, n = 6) on WAY 100135 (50 μM, n = 10)-induced increase in glutamate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P <$ 0.05.

Fig 6,15



Effect of omitting Ca^{2+} from the dialysis fluid (from moment of implantation, n = 6) on the effect of WAY 100135 (50 μM, n = 10 on aspartate concentration in striatal dialysate. Data were analysed with Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. No significant differences were observed.

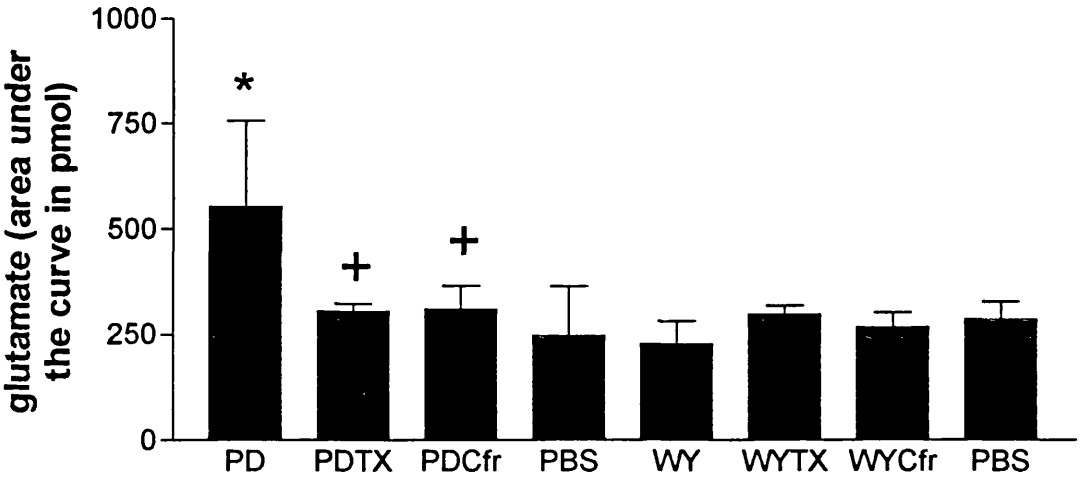
Fig 6,17



Effect of drugs topically applied to the frontal cortex on ASP concentrations in striatal dialysate, expressed as area under the curve (pmol). Data were analysed using a Kruskal-Wallis ANOVA followed by either a Mann Whitney U test or a LSD test as appropriate. No significant differences were observed.

(PBS = phosphate buffered saline; PD = PD142505-0028 10 μM; PDTX = PD 142505-0028 + tetrodotoxin 10 μM; PDCafr = PD 142505-0028, dialysis with Ca^{2+}-free medium; WY = WAY 100135 50 μM; WYTX = WAY 100135 50 μm + tetrodotoxin 10 μM; WYCfr = WAY 100135 50 μM, dialysis with Ca^{2+}-free medium).

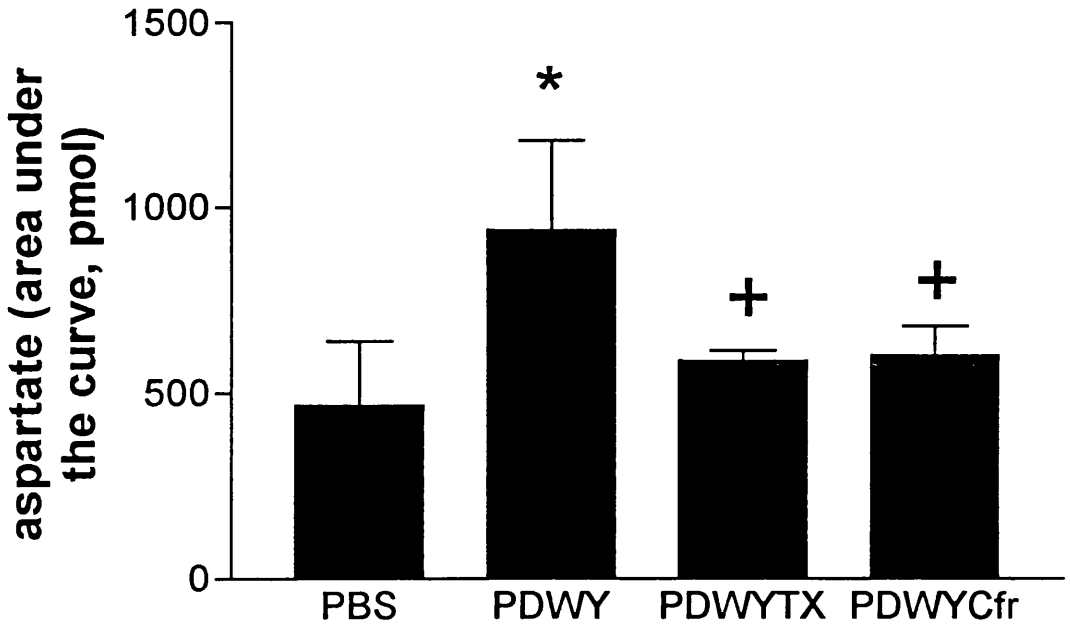
Fig 6,18



Effect of drugs topically applied to the frontal cortex on GLU concentrations in striatal dialysate, expressed as area under the curve (pmol). Data were analysed using a Kruskal-Wallis ANOVA followed by either a Mann Whitney U test or a LSD test as appropriate. * = $P < 0.05$ as compared to vehicle, + = $P < 0.05$ as compared to the PD + effect.

(PBS = phosphate buffered saline; PD = PD142505-0028 10 μM; PDTX = PD 142505-0028 + tetrodotoxin 10 μM; PDCafr = PD 142505-0028, dialysis with Ca^{2+}-free medium; WY = WAY 100135 50 μM; WYTX = WAY 100135 50 μm + tetrodotoxin 10 μM; WYCfr = WAY 100135 50 μM, dialysis with Ca^{2+}-free medium).

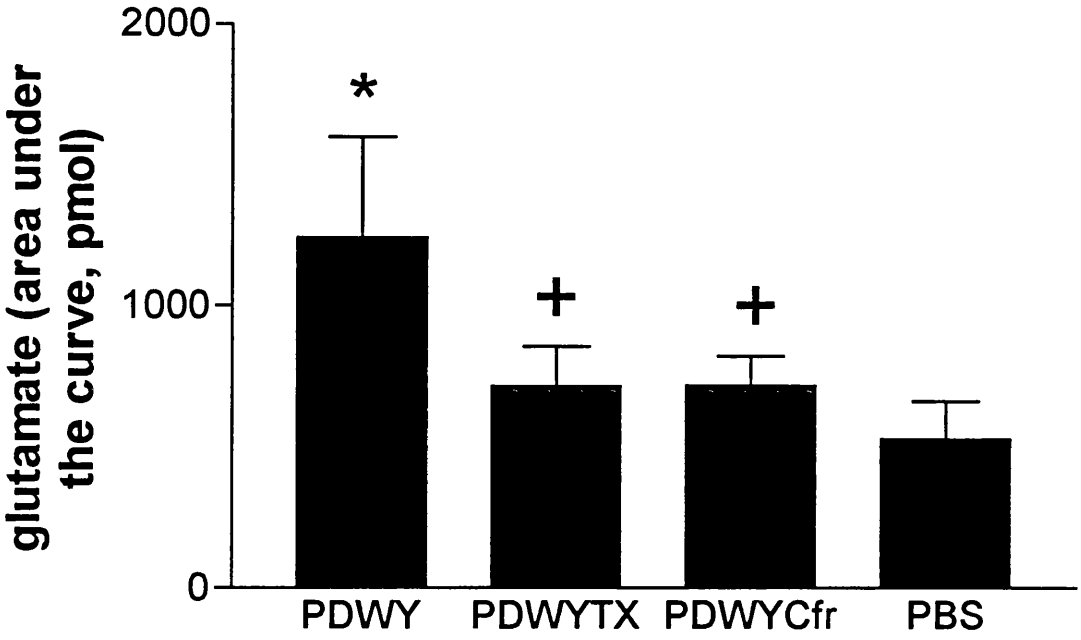
Fig 6,19



Effect of drugs topically applied to the frontal cortex on ASP concentrations in striatal dialysate, expressed as area under the curve (pmol). Data were analysed using a Kruskal-Wallis ANOVA followed by either a Mann Whitney U test or a LSD test as appropriate. * = $P < 0.05$ as compared to vehicle, + = $P < 0.05$ as compared to the PD + WAY effect.

(PDWY = PD 142505-0028 10 μM + WAY 100135 50 μM; PDWYTX = PD 142505-0028 + WAY100135 50 μM + tetrodotoxin 10 μM; PDWYCafr = PD 142505-0028 10 μM + WAY 100135 50 μM, dialysis in Ca^{2+}-free medium; PBS = phosphate buffered saline).

Fig 6,20



Effect of drugs topically applied to the frontal cortex on GLU concentrations in striatal dialysate, expressed as area under the curve (pmol). Data were analysed using a Kruskal-Wallis ANOVA followed by either a Mann Whitney U test or a LSD test as appropriate. * = $P < 0.05$ as compared to vehicle, + = $P < 0.05$ as compared to the PD + WAY effect.

(PDWY = PD 142505-0028 10 μM + WAY 100135 50 μM; PDWYTX = PD 142505-0028 + WAY100135 50 μM + tetrodotoxin 10 μM; PDWYCafr = PD 142505-0028 10 μM + WAY 100135 50 μM, dialysis in Ca^{2+}-free medium; PBS = phosphate buffered saline).

6.4 Discussion

The present results suggest that increased activity of corticostriatal pyramidal neurones, induced by a selective M_1 agonist can be potentiated by a selective 5-HT_{1A} antagonist.

The important contribution of ACh in regulation of functional levels of neuronal excitability has been studied extensively (for review see: Ashe & Weinberger, 1991), In sensory neocortex, ACh has been shown to modify neuronal responsiveness to sensori stimuli (Silito & Kemp, 1983; McKenna et al., 1988; Metherate et al., 1988a,b) and these changes in neuronal responses can be relatively long in duration and can result in a lasting modification of the receptive field of a neurone. In addition, nucleus basalis stimulation elicits EEG activation and produces a shift in subtreshold membrane potential fluctuations from large-amplitude, slow (1-5 Hz) oscillations to low-amplitude, fast (20-40 Hz) oscillations, a shift in pattern of membrane potential fluctuations that results in change of spike discharge pattern from phasic to tonic (Metherate & Ashe,1993). All of these actions are blocked by atropine, suggesting involvement of muscarinic receptors.

In recent years the mode of action by which acetylcholine increases the excitability of cortical pyramidal neurones has become clearer, especially from *in vitro* studies using slices or isolated neurones. These studies show that activation of muscarinic cholinergic receptors in the CNS initiates a variety of intracellular processes, such as modulation of second messenger levels and ion channel activity (Nicoll et al., 1990). Electrophysiological studies for example in hippocampal neurones have established that stimulation of postsynaptic muscarinic receptors leads to a reduction in membrane potassium conductances (Dodd et al., 1981). This modulation by M_1 receptor agonists of potassium channels appears to involve a G-protein coupled mechanism. Although little is known about the mechanisms by which second messenger pathways link these receptors to the modulation of a defined K^+ channel, a recent paper (Peralta, 1995) provides evidence that M_1 agonists suppress a delayed rectifier potassium channel through the PLC pathway by direct

tyrosine phosphorylation of the protein by PKC.

The selective 5-HT_{1A}-antagonist WAY 100135 increased slightly, but significantly basal concentrations of striatal glutamate. The most straightforward explanation for this effect is that the compound reduces the resting potential of cortical pyramidal neurones, by blocking the action of 5-HT, an hyperpolarising transmitter, thereby increasing the likelihood that a given cell is depolarised by endogenous excitatory amino acids. Using an *in vitro* brain slice preparation Andrade (1992) has shown that 5-HT_{1A} agonists elicit a membrane hyperpolarisation that effectively inhibits neuronal activity. This effect is dependent on intracellular GTP, suggesting the involvement of a G protein. Indeed the stable GTP analogue GTPγS renders the serotonin induced hyperpolarisation irreversible. The 5-HT_{1A} receptor induced hyperpolarisation is mediated by an increase in potassium conductance. While the potassium channel needs to be identified, it may be an inwardly rectifying one (Andrade, 1992).

CHAPTER 7

Preliminary evidence that elevating extracellular potassium concentration increases 22C11 immunoreactivity in striatal push-pull perfusate.

7.1 Introduction

Hypoactivity of cortical pyramidal neurones has been hypothesised to underlie not only the cognitive symptoms of Alzheimer's disease (AD), but also to accelerate the formation of the two histological hallmarks the senile plaque and the neurofibrillary tangle (Francis et al., 1993).

Recent *in vitro* studies (e.g. Nitsch et al., 1994) have suggested that an increased neuronal activity stimulates secretion of amyloid precursor protein, while other studies (Jacobsen et al., 1994) have suggested that an increased secretion is accompanied by a parallel reduction in the formation of ßA4.

In previous Chapters the effect of a selective M_1 agonist (Chapter 5) and a selective 5-HT_{1A}-antagonist (Chapter 6) on activity of cortical pyramidal neurones has been detailed.

In this preliminary study the central question addressed was whether an increase in neuronal activity, common denominator of drugs previously investigated, would stimulate secretion of amyloid precursor protein in an *in vivo* setting, using the push-pull technique in the anaesthetised rat.

7.2 Materials and Methods.

Western blotting and the *N*-terminal antibody 22C11 were used to assay the samples for APP content.

7.2.1 Animals and surgery

All procedures were carried out under appropriate personal and project licences under the

Animals (Scientific Procedures) Act 1986. Male Sprague Dawley rats (Charles River, U.K) weighing between 200 and 250 grams were housed individually and had free access to food and water with a twelve hour light/dark cycle (lights on 0700h). Humidity and temperature were maintained at 45-50% and 20-21 °C, respectively. Before the experiments animals were allowed a one week period of adaptation. At the start of the experiment rats were anaesthetised with halothane in a mixture of oxygen and nitrous oxide until no reaction to tail and hind paw pinch was detectable. Under full anaesthesia animals were mounted in a Kopf stereotactic frame and a burr hole was made over the striatum to allow implantation of the push pull probe (coordinates with bregma as reference: AP 0 mm, L 2.5 mm, DV 7 mm from skull).

7.2.2 Push-pull and experimental set-up.

A push-pull probe was attached to a Watson-Marlow pump (Falmouth, Cornwall, UK). In and outflow were calibrated prior to starting the experiment. Typical flowrates were between 8 and 10 μl/min. After implantation the probe was perfused with a Ringer solution (containing in mM NaCl 141; $MgCl_2$ 1.3; $CaCl_2$ 1.3; KCl 5). Collection time per sample was 30 minutes. After a stabilisation period of three samples (1.5 hours), tissue around the probe was perfused with Ringer containing 100 mM K^+ for five more samples (the perfusate in these experiments contained in mM: NaCl 46; $MgCl_2$ 1.3; $CaCl_2$ 1.3; KCl 100; n=6). In 4 control experiments normal Ringer was used for the whole experiment.

7.2.3 Western blotting and quantification of APP.

Push-pull samples were collected in Amicon Centricon-30 tubes. Samples were stored on ice, 50 μl was taken out of the sample, deproteinised with acidified methanol and stored in - 70 °C

until analysed for amino acid content (see section 2.6). The remainder of the sample was concentrated to a final volume of 50 μl. Samples were analysed for ßAPP-like immunoreactivity using SDS-PAGE according to Webster et al. 1994, and the ßAPP amino terminal antibody 22C11 (epitope mapped to amino acids 60-100; Boehringer Mannheim, Lewis, UK) (see section 2.7). Immunoreactive proteins were visualised (ECL kit; Amersham International) and scanned with a densitometer (EDC densitometer; Helena labs). The integral of total band density for each sample was divided by that of background. These values were then meaned.

7.2.4 Statistical analysis

The variance of data obtained following increasing extracellular potassium concentrations was significantly different from control, therefore the Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test was used to compare individual time points. The null hypothesis was rejected when $P < 0.05$.

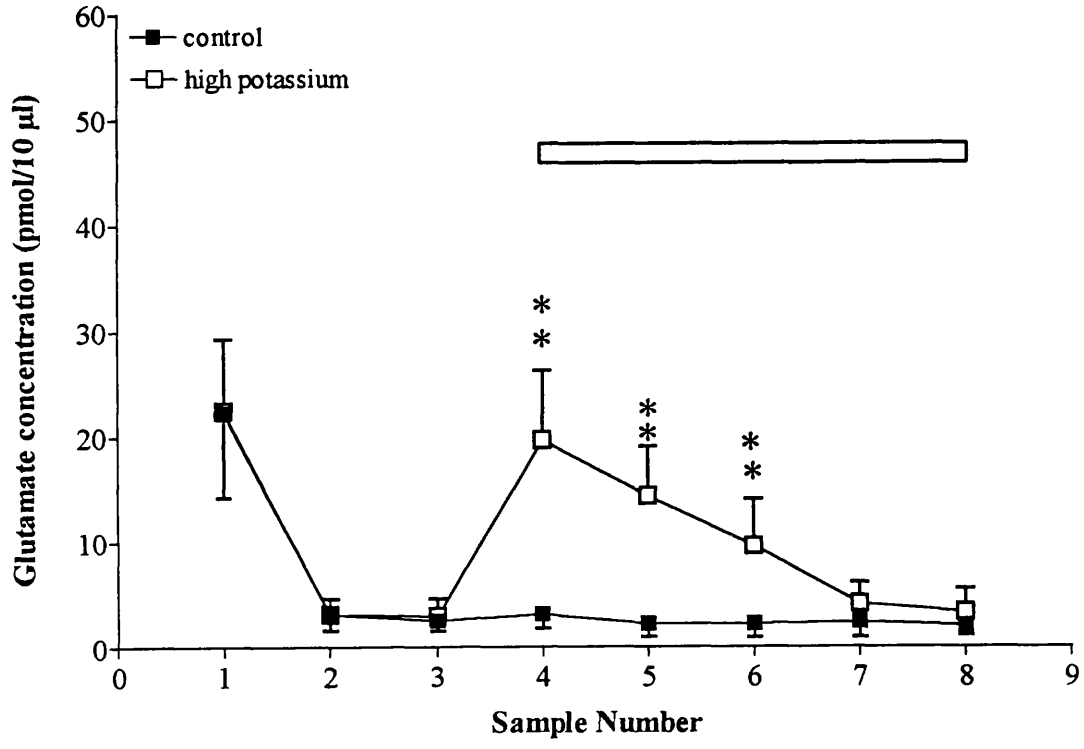
7.3 Results

Elevation of extracellular potassium in the perfusion fluid (Figure 7,1 bar) resulted in a significant increase in the concentration of glutamate (Fig. 7,1, open square, n = 6) as compared to control (Fig. 7,1 filled squares, n =4).

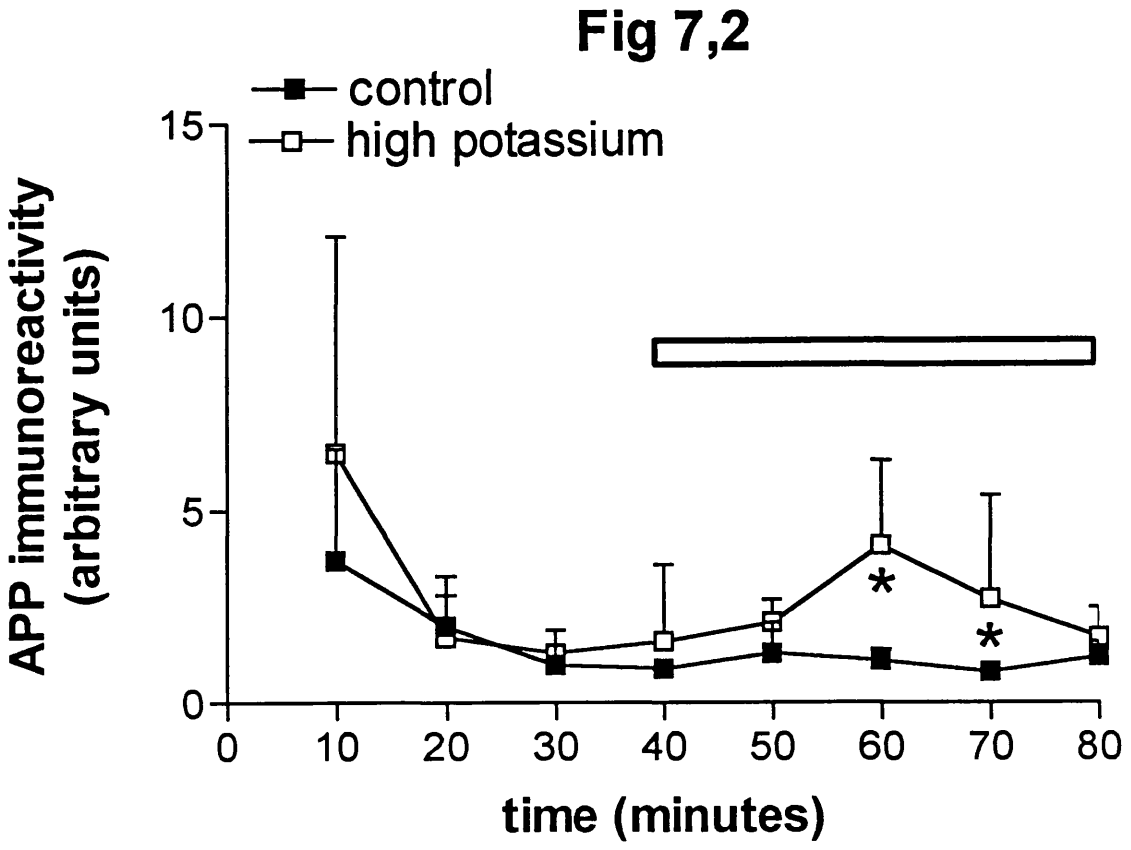
Elevating potassium concentrations increased ßAPP total immunoreactivity significantly starting from the fifth until the seventh sample (Fig. 7,2, filled triangle, n = 6, $P < 0.05$), while perfusion with normal Ringer failed to affect ßAPP immunoreactivity (Figure 7,2, open squares, n = 4).

In a typical experiment 22C11 recognised two major bands of ßAPP-like immunoreactivity in the 116-140 kDa range, while minor bands were sometimes present (see Fig. 7,3).

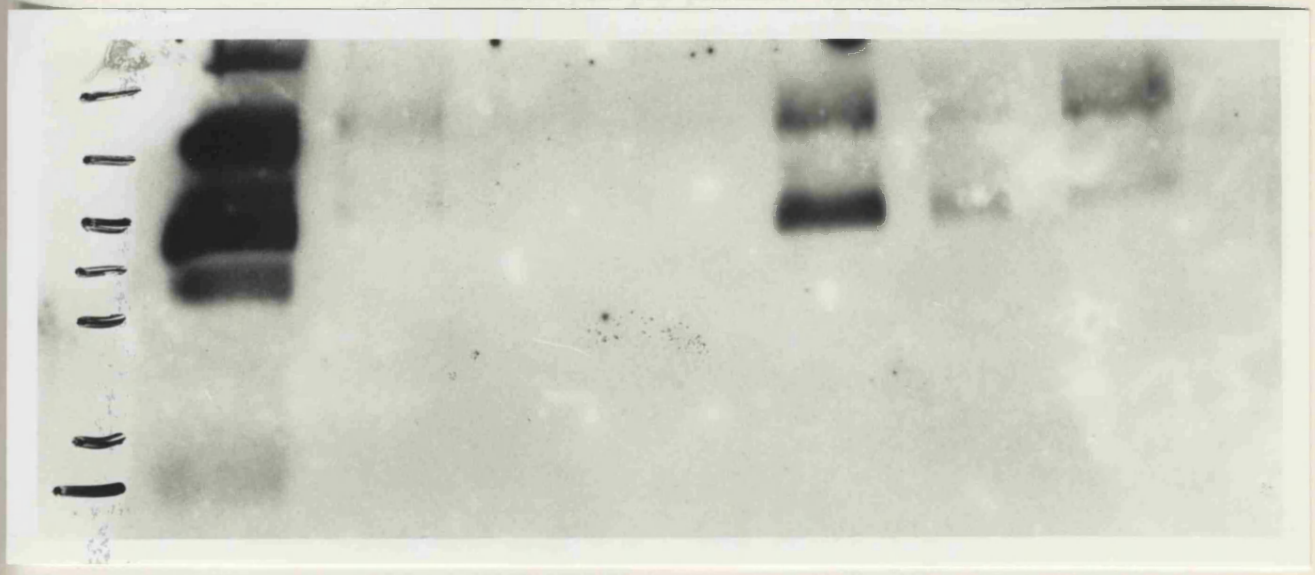
Figure 7,1



Effect of elevation of extracellular potassium on glutamate concentration in striatal push-pull perfusate (bar, 100 mM, n = 6) as compared to control (n = 4). Data were analysed using Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. ** = $P < 0.01$.



Effect of elevation of extracellular potassium on 22C11 immunoreactivity in striatal push-pull perfusate (bar, 100 mM, n = 6) as compared to control (n = 4). Data were analysed using Kruskal-Wallis ANOVA followed by the Mann-Whitney-U test. * = $P < 0.05$.



Photograph of a typical Western blot. The first sample (on the left) is where the probe is implanted in the striatum. 22C11 immunoreactivity reappears in the fifth sample, and continues to be visible in sample 6 and 7.

7.4 Discussion

The results described in this Chapter suggest that elevation of extracellular potassium concentration increased extracellular glutamate. Therefore it seems likely that depolarisation of neurones around the push-pull probe had occurred. This was associated with an increase in APP-like immunoreactivity in rat striatal push-pull perfusate. Since the antibody 22C11 does not distinguish between APP and the APLP proteins, future studies will need to resolve the relative contribution of APP and the APP-like proteins APLP1 and/or 2. Similarly the effect of an increased metabolism of the APP protein through the secretory pathway on formation of Aß *in vivo* needs further study. Some *in vitro* studies suggest that increasing secretion of ßAPP can be accompanied by a concomitant reduction of the formation of ßA4 (Fukushima et al., 1993; Jacobsen et al., 1994).

In vitro studies have shown that both electrical depolarisation (Nitsch et al., 1993) and stimulation of M_1-receptors (Nitsch et al., 1992) increase secretion of APP, while the acetylcholinesterase inhibitor physostigmine increases APP secretion in cortical slices (Mori et al.,1994) through the M_1 receptor. These findings link in with the finding described in Chapter 5 that physostigmine increased the activity of cortical pyramidal neurones through the M_1 receptor. It therefore follows that such drugs may increase APP secretion *in vivo* via increasing neuronal activity.

In AD hypoactivity of cortical pyramidal neurones may not only accelerate the formation of plaques by favouring amyloidogenic metabolic processing of APP, but a reduced secretion of APP may accelerate neuronal death if a vital function of the peptide is lost. In both instances stimulation of secretion of APP by increasing neuronal activity may be beneficial.

Chapter 8

General Discussion

8.1 Loss of pyramidal neurones from frontal and temporal cortex is a seminal event in the pathology of AD.

Pyramidal cell loss, both from cortex and hippocampus is a seminal event in the development of AD. For example, in a study by Mann et al., (1988) on biopsy and autopsy samples taken from brains from patients suffering from AD, the density and nucleolar volume of pyramidal nerve cells was significantly less than controls, and, in gneeral, values for both these measures fell significantly further from biopsy to death. By contrast, in none of the patients studied did senile plaque density consistently change rfrom biopsy to death. Also, neurofibrillary tangle density either did not change, or indeed sometimes decreased from biopsy to death. Thus the pathological progression of AD is marked by a continuing loss of pyramidal cells from frontal and temporal cortex. Furthermore, the extent of pyramidal nerve cell loss in termporal cortex correlates strongly with the degree of dementia (Neary et al., 1986). Thus the studies performed in this thesis have addressed the question how the activity of cortical pyramidal neurones can be modulated, using topical application of drugs to the frontal cortex, and striatal microdialysis of glutamate and aspartate coupled to High Performance Liquid Chromatography with OPA derivitisation to follow the release of EAA's in the terminal field of the corticostriatal pathway.

8.2 Differential effects of drugs on ASP versus GLU release from corticostriatal neurones.

The main findings of this thesis are that, when topically applied to the frontal cortex, NMDA, the selective 5-HT_{1A}- antagonist WAY 100135, the M_1 -agonist PD 142505-0028 and, when injected intramuscularly, the acetylcholinesterase inhibitor physostigmine all increase concentrations of glutamate in the striatal dialysate. NMDA and the combination of the M_1

agonist PD 142505-0028 plus the selective 5-HT_{1A} antagonist WAY100135 increased concentrations of aspartate. When analysing the data as percentage increase of baseline, and when the area under the curve is calculated, NMDA released relatively more ASP than GLU. The reason for this is unclear, but glucose availability may play a role. It is possible that NMDA may have overstimulated cortical pyramidal neurones, when topically applied to the frontal cortex, and in doing so may have lowered extracellular concentrations of glucose. That low extracellular glucose affects the relative ratios of ASP versus GLU release has been documented by Szerb (1988). In this study the stimulus-induced decrease in GLU content and release in hippocampal slices, when extracellular glucose was lowered, was hypothesised to be the result of a decreased synthesis of new glutamate, which would normally replace the released transmitter. Not only does a lowered extracellular glucose results in a deficiency of the main precursor for glutamate synthesis, but indirectly, low-glucose-containing medium restricts the availability of its other precursor, glutamine, by increasing its utilisation as a substrate for oxidation (Tildon, 1983). Low glucose, while inhibiting the synthesis of glutamate, enhances that of aspartate, because oxaloacetate, the immediate precursor of ASP, accumulates when there is insufficient amounts of acetyl-CoA to form citric acid (Haslam & Krebs, 1963). The net effect of reduced glucose availability is a reduced synthesis of glutamate due to the insufficient formation of its precursors, while increasing the synthesis of ASP, due to accumulation of its precursor, oxaloacetate.

The NMDA-evoked release of both ASP and GLU was Ca^{2+}-dependent and tetrodotoxin sensitive, strongly suggesting that the release originated from nerve terminals, depolarised by action potentials. In addition to this it suggests a neurotransmitter role for both ASP and GLU in the striatum, a situation which would be similar to, for example the hippocampus (see Fleck et al., 1993)

In the experiments described in Chapter 3,4,5 and 6 a differential effect of drugs on ASP versus GLU release was observed. For example, WAY 100135, PD 142505-0028 and

physostigmine all induced an increase in GLU concentrations in striatal dialysate, (although in the analysis using the area under the curve the effect of WAY 100135 lost its significancy), but not in ASP concentrations. One possible explanation for this finding could be that in the EAA terminal of the corticostriatal pathway ASP release is differentially coupled to second messenger systems as compared to GLU release, resulting in different release profiles. Alternatively, the corticostriatal pathway may consist of different subsets of neurones, all with a slightly different receptor repertoire, or, again, with different coupling of receptors to second messenger systems.

8.3 Does microdialysis measure the true extracellular concentration of a substance?

Several studies have addressed the question whether the dialysis probe can determine the actual concentration of a substance in the extracellular space (for review see: Benveniste & Huttemeier, 1989). Earlier studies tended to determine the *in vitro* recovery and calculate from this value, together with the value measured in the dialysis fluid, the actual concentration. This is now considered incorrect because of the difference in the diffusion characteristics of any given substance in an *in vitro* situation compared with an *in vivo* situation.

In general terms three approaches have been used to determine the true extracellular concentration in the extracellular space. The first was introduced by Jacobsen et al., (1985) who, in an *in vivo* setting, varied the flow rate through the dialysis probe, and extrapolated to zero flow, a situation where the concentration in and outside the probe will be the same. The second approach was introduced by Lonnroth et al., (1987), who determined the equilibrium constant of the substance of interest by perfusing different concentrations through the dialysis probe and then calculating the concentration at which the substance in the perfusate does not change during the perfusion, because it has the same concentration inside the probe as in the extracellular fluid. A third approach is to use a reference substance and the observation that recovery over the membrane of a given substance is identical in both directions (Amberg & Lindefors, 1989).

Measurement of the amount of reference substance lost from the dialysis fluid gives an indication of its recovery, and this will be the same as the recovery of the substance of interest if the substances have similar diffusion characteristics.

All of the approaches mentioned make the tacit assumption that the diffusion characteristics of the substance of interest remains constant throughout the experiment. If this alters during the experiment the resulting change in the concentration of the compound in the dialysis fluid could be interpreted as a change in release. A change in diffusion characteristics can, for example, be caused by development of oedema in the tissue around the probe (Benveniste & Huttemeier, 1989). One way to control for this is by including a reference substance in the dialysis fluid. A change in the concentration of this substance during the experiment would indicate a change in diffusion coefficient and this can than be used as a correction factor.

8.4 Comparison of push-pull and microdialysis

The main difference between a push-pull perfusion probe and microdialysis probe is the presence of a dialysis membrane. The presence of this membrane has several advantages. First, because of the fact that the dialysis membrane acts as a mechanical barrier, turbulence in the fluid flow, which can cause brain tissue damage, is reduced. High flow rates (10-20 μl/min) are normally required to prevent blockage of fluid flow, but these flows can cause significant damage. Because this problem does not exist in microdialysis, chance of tissue damage is also reduced. One question which is important in all microdialysis studies, but which gains extra importance when push pull is used to study the release of neurotransmitters is what part of the outflow measured is related to neuronal activity and what is due to neuronal damage. Parameters used to address this question is tetrodotoxin- sensitivity and Ca^{2+}-dependency. Data on the Ca^{2+}-dependency and the tetrodotoxin-sensitivity using the push-pull technique are scarce in the

literature. However, in cats, infusion of tetrodotoxin only partly inhibited the release of [^{3}H] dopamine from the striatum (Nieoullon et al., 1977). Taken together it is likely that push-pull perfusion causes more damage to neuronal tissue than the microdialysis method, and may not be the method of choice for studying neurotransmitters.

Technically push-pull perfusion is more difficult than microdialysis because of the need to exactly balance the amount of fluid going into the brain to that being withdrawn from it. Buildup of back-pressure and clogging in the push pull probe may occur, especially when smaller cannulas (0.5 mm outer diameter) are used. When lower flow speeds are used clogging occurs frequently.

There are certain situations in which only push-pull cannulas can be used. The absence of a membrane barrier means that molecules with high molecular weight, which would be excluded by the dialysis membrane, can be measured.

8.5 Possible confounding presynaptic mechanisms.

Under the present experimental conditions it is difficult to ascertain whether drugs topically applied to the frontal cortex interact directly with receptors on cell bodies and/or dendrites of pyramidal neurones or whether release of either inhibitory or excitatory neurotransmitters from presynaptic terminals determine the final outcome of the experiment. This question is also linked to the fact that halothane is used for anaesthesia. Although the mechanism of action of volatile anaesthetics is complex, one feature seems to be that they increase GABA-ergic tone, especially through the GABA-A receptor (Longoni et al., 1993). Recent studies have identified heteroreceptors on cortical GABA-ergic interneurones (Hashimoto et al., 1994), which influence their activity. The M_1 receptor especially seems important for the regulation of activity of these interneurones, in that activation of this receptor subtype *decreases* GABA release. Another study focused on the effect of an AMPA lesion of the nucleus basalis magnocellularis, on GABA-ergic

tone in the frontal cortex of the rat (Abdulla et al., 1994). An increased GABA-ergic tone, six to eight weeks after the lesion, was observed. Thus, an increased GABA-ergic tone may be a feature of both AD, and of halothane anaesthesia, and at least this aspect of the disease may be mimicked under the present experimental conditions. Topical application of M_1 agonists, and peripheral injections of physostigmine may therefore increase cortical pyramidal neurone activity through direct activation of M_1 receptors on cell bodies and/or dendrites, and by activation of M_1 heteroreceptors on GABA-ergic interneurones. In addition, GABA-ergic interneurones are resistant both to NMDA receptor mediated injury (Tecoma & Choi, 1989), and are also resistant to toxicity induced by ß-amyloid (Pike & Cotman, 1993), so they may survive longer during the disease process, and contribute to inhibitory tone on cortical pyramidal neurones.

A second possible way in which presynaptic mechanisms have influenced activity of cortical pyramidal neurones under the experimental conditions described in this thesis is through an interaction between 5-HT and ACh release in the frontal cortex. Most previous studies on this subject point to an inhibitory tone of 5-HT on ACH release in the cortex (e.g. Vizi et al., 1981; Gillet et al., 1985; Jackson et al., 1988), a hypothesis supported by the finding of Robinson, (1983) that lesions of the raphe nuclei enhance ACh turnover, amongst others in the cortex. However, a recent study focused on the effect of the 5-HT releasing compound fenfluramine on ACh release in the cortex, (without inclusion of an acetylcholinesterase inhibitor in the dialysis fluid) and observed a dose dependent stimulatory effect, which was blocked in part by the $5HT_{2A}$ antagonist ketanserin (Hirano et al., 1995). Although the localisation of $5HT_{2A}$ receptors remains controversial, some studies point to a localisation on terminals of cortically projecting cholinergic neurones (Quirion et al., 1985), which would link in with the excitatory influence of 5-HT on ACh release. In AD, cortical $5HT_2$ receptors have been reported to be decreased (Cross et al., 1984a), and this could compound the cholinergic deficit characteristic of this disease.

In the experiments using NMDA a third possible confounding presynaptic mechanism may

have contributed to the final result. Under the present experimental conditions it can not be ruled out that NMDA, when topically applied to the frontal cortex, may have diffused directly into the striatum and altered excitability of the corticostriatal terminals (see for example: Garcia-Munoz, 1991)

Because it is difficult to separate presynaptic modulation of topically applied drugs from a direct interaction of drugs with receptors on cell bodies and/or dendrites of cortical pyramidal neurones, careful evaluation of the effect of these drugs when given peripherally will need to done.

8.6 Will increasing neuronal activity increase the vulnerability of remaining neurones to excitotoxicity?

In Alzheimer's disease, hypoactivity of cortical pyramidal neurones may underlie both the cognitive symptoms and may accelerate the development of the histological hallmarks. If drugs are tried which increase this hypothesised hypoactivity, on side-effect may be excitotoxicity. (see section 1.2.5). Although careful evaluation of any therapy will need to consider this possibility, some aspects of AD suggest that increasing glutamate concentrations may not automatically endanger remaining neurones. Although in general a reduced glucose utilisation is considered to speed up the pathogenesis of AD (see for example: Hoyer, 1993). For example, glucose appears to be essential for the formation of ACh (Gibson & Blass, 1976; Sims et al., 1981), and energy in the form of ATP (Erecinska & Silver, 1989; Hoyer, 1992). ATP is essential for synaptic transmission (see for example Huganir & Greengard, 1990), and for maintaining ionic homeostasis (Siesjö, 1981). On the other hand, reduced availability of glucose may in effect be a protective mechanism against excessive released glutamate. Although future studies will need to address this issue in more detail, a study which combined hypoxia with ischemia, reducing plasma glucose concentrations in the low physiological range markedly

protected the striatum against the development of damage, even though glutamate was still released into the extracellular space in large quantities (Dijk et al., 1994).

In another development it has been proposed that stimulation of for example G-protein linked muscarinic receptors may ultimately result in development of neuronal damage (see Olney, 1994). Although cholinomimetic therapy would stimulate this class of second messengers, combination therapy with a 5-HT_{1A} receptor antagonist may counteract this effect. Additionally evidence is emerging that the delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis (Nitatori et al., 1995) and that in AD apoptosis may be triggered untimely in the adult CNS (Barinaga, 1993). The development of apoptosis seems to be dependent on many factors, but activation of muscarinic M_3 receptors seems to block apoptosis, at least in cultured cerebellar granule neurones (Yan et al., 1995). In this manner cholinomimetics may counteract the development of cell death in AD.

8.7 Does increased neuronal activity slow down the development of the histological hallmarks of AD?

The *post-mortem* brain of the AD patient is characterised by the neurofibrillary tangle and the senile plaque. Although future studies will need to address in more detail the influence of neuronal activity on the development of these structures, there are some studies which suggest that increased neuronal activity may slow down the development of these histological hallmarks. PHF tau formation is associated with an abnormal tau hyperphosphorylation state. It is unknown whether and if, in what way neuronal activity will affect tau hyperphosphorylation, but as the mechanism is unknown it may be pessimistically speculated that this pathology would appear with agents stimulating the M_1 receptor, and consequent PKC activation. However, there is little evidence that this is the case (e.g. Goode et al., 1992 and section 1.2.3). For example, when briefly treated with glutamate, cortical neurones contained much lower amounts of PHF-like tau

than in the control condition (Brion et al., 1993; Davis et al., 1994). The second structure which is typical of AD is the senile plaque, the main constituent of which is ßA4 (see section 1.2.2). As discussed in section 1.4, increasing neuronal activity may be accompanied by an increased secretion of APP, which in turn may result in stimulating the metabolism of APP through the non-amyloidogenic pathway. This would not only slow down the development of senile plaques, but as there is evidence that ßA4 can be neurotoxic (see section) such treatment may indirectly protect remaining neurones. Additionally a recent study suggests that ßA4 may indirectly increase the formation of hyperphosphorylated tau (Busciglio et al., 1995), and reducing the formation of ßA4 may thus slow down tau becoming hyperphosphorylated.

Another view is that increasing the secretion of APP will restore an essential function of the protein, which is lost in AD brain because hypoactivity of neurones decreases secretion. That APP can be beneficial to neurones has been shown (e.g. Mattson et al., 1993; Smith-Swintovsky et al., 1994; Yamamoto et al, 1994), while another study shows that the trophic domain of the APP protein can increase synaptic density in the frontoparietal cortex, while increasing memory retention Roch et al., 1994). This study thus concludes that APP is involved in memory retention processes, probably through its effect on synaptic structure. The conclusion which may be drawn from these studies is that it may be beneficial to the AD brain to increase secretion of APP, maybe by increasing neuronal activity.

8.8 What are the possible side effects of treatment with a 5-HT_{IA} antagonist?

An impressive corpus of data has accumulated over the last 15 years, pointing toward the 5-HT system as very important in mediating the therapeutic effect of antidepressant therapy (see for example: Blier et al., 1990), and especially the 5-HT_{1A} receptor seems pivotal in the development of affective and anxiety disorders. Selective 5-HT_{1A} agonists have been proposed as being beneficial both in affective and anxiety disorders (see: de Montigny & Blier, 1992), and

it could therefore be argued that treatment of AD patients with a selective 5-HT_{1A} antagonist could result in these patients becoming either anxious or depressed or both. Obviously careful evaluation of any treatment needs to be carried out, but the situation concerning the 5-HT system in AD is complex, and a case can be made for 5-HT_{1A} antagonists. First antidepressant therapy may be only become effective when a significant enhancement of 5-HT neurotransmission takes place. If a selective 5-HT_{1A} antagonist, by blocking autoreceptors on cell bodies in the dorsal raphe, enhances serotonergic tone in the cortex, this may mediate an antidepressant/anti-anxiety effect. Indeed there is evidence in the literature that WAY 100135 can be anxiolytic (Rodgers & Cole, 1994). That increased serotonergic neurotransmission is part of antidepressant therapy is indirectly supported by a recent study which focused on the status of the serotonergic system in patient suffering from AD with and without depression. In all patients loss of dorsal raphe neurones was observed, but presynaptic serotonergic markers ([^{3}H]paroxetine binding (a putative marker of 5-HT terminals), and 5-HT and 5-HIAA concentration) in the cortex were only significantly different from control in patients suffering from depression (Chen et al., 1996) It is tempting to hypothesise that an increased serotonergic tone in one subgroup of AD patients has protected them from developing behavioural symptoms, like depression and anxiety . Previous reports suggest that in AD the concentration of 5-HT is reduced in most but not all brain regions (as reviewed: Palmer et al. 1987a, Palmer et al., 1988, Francis et al., 1993a). A potential explanation for this difference is that chronic neuroleptic medication may have influenced the 5-HT and 5-HIAA concentrations. Indeed in the study by Chen et al. 5-HT and 5-HIAA in a subgroup of AD patients which had received chronic neuroleptic treatment were lower than in patients who had not undergone such treatment. Previous studies may have inadvertently used patients who had been institutionalised, and who were likely to have been on neuroleptic medication.

In conclusion a careful evaluation of treatment of AD patients with a 5-HT_{1A} antagonist should be done, but trials using this class of drug should not be withheld because of potential

side effect on mood.

8.9 Future studies

Future studies may investigate drugs acting at other receptors in the topical application model. Interesting possibilities would include drugs acting at glutamate metabotropic receptors, 5-$HT_{2A/2C}$ receptors, 5-HT_{1D} receptors.

New drugs acting either as cholinesterase inhibitor or M_1 agonist could be tested.

The model should be developed further in that more aspects of AD could be mimicked. For example, a partial dorsal raphe lesion could be combined with a nbM lesion.

Drugs should also be tested in the lesioned freely moving animal.

An interesting experiment would be to investigate whether M_1 agonists could still enhance cortical pyramidal neurone activity in the presence of a cocktail of GABA antagonists.

The push-pull method should be developed further. The influence of neuronal activity on the release of amyloid precursor like proteins should be investigated. Another interesting experiment would look at whether cholinesterase inhibitors can increase APP release.

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