The Effect of Anaesthetic and Analgesic Agents on Glutamate Uptake and Release from Rat Neuronal Preparations

By

Beverley Nicol BSc (Hons)

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The effect of anaesthetic and analgesic agents on glutamate uptake and release from rat neuronal preparations.

Beverley Nicol.

Abstract

Glutamate is the principal excitatory neurotransmitter within the CNS. Depression of excitatory transmission may produce anaesthesia. After primary impact injury, ischaemic events or haematomas, it has been shown that either the excessive release of glutamate or the inhibition of the uptake, may be responsible for the excitotoxic damage to neurones and glia.

In rat cerebrocortical and cerebellar synaptosomes the uptake of [^{3}H]L-glutamate was time-, concentration-(K_m=~8.6 and ~2.2μM respectively), Na^+- dependent and L-trans-PDC sensitive. In general the uptake mechanism was insensitive to clinically relevant concentrations of anaesthetics.

Perfused rat cerebrocortical slices depolarised with 2×2min pulses of 46mM K^+ (S_1 and S_2) evoked monophasic releases of endogenous glutamate with an S_2/S_1 ratio of ~1.07. 0.1mM EGTA pre-treatment significantly inhibited evoked release, confirming a Ca^{2+} regulated exocytotic process.

A range of μ and κ, but not δ ligands inhibited evoked release of glutamate (e.g. morphine IC_{50} 71nM). A novel heptadecapeptide, nociceptin produced a dose-dependent naloxone insensitive inhibition of evoked glutamate release (IC_{50} of 51nM). The anaesthetic agents propofol, thiopentone, ketamine and halothane, dose-dependently inhibited the evoked release of glutamate with IC_{50}s of ~20, 10, 18 and 132μM respectively. At single doses 1μM MK-801, 100μM pentobarbital, 435μM isoflurane significantly inhibited release. A non-anaesthetic barbiturate, barbituric acid was ineffective. These data suggest that glutamate release may be a target for anaesthetic agents.

[^{3}H]MK-801 bound to NMDA receptors in a time- and concentration-dependent manner (K_D=~3nM, B_{max}=~900fmoles/mg protein). The racemic mixture, R- and S+ isomers of ketamine significantly displaced the binding with estimated K_i values of ~638, 2703 and 568nM respectively. Etomidate significantly displaced the binding of [^{3}H]MK-801 with an estimated K_i of 4μM. Propofol, thiopentone and pentobarbital, barbituric acid, halothane and morphine were ineffective. Block of pre-synaptic NMDA receptors alone cannot explain the observed anaesthetic/analgesic inhibition of evoked glutamate release.

Collectively these data support a role for glutamatergic neurotransmission in the mechanism of anaesthesia/analgesia and may implicate anaesthetic/analgesic agents as neuroprotectants.

Acknowledgements

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Abbreviations

AC: adenylate cyclase.

cAMP: cyclic adenosine monophosphate.

AMPA: α-amino-3-hydroxy-5-ethyl-4-isoxazole proprionic acid.

AP: action potential.

AP_4: 2-amino-4-phosphonobutyrate.

AP_7: 2-amino-7-phosphonobutyrate.

ATP: adenosine triphosphate.

ATPase: adenosine triphosphatase.

nor-BNI: norbinaltorphimine.

BSA: bovine serum albumin.

Ca^{2+}: calcium.

$[Ca^{2+}]_i$: intracellular calcium concentration.

Cb: cerebellar.

CCh: carbachol.

CHO: chinese hamster ovary.

Cl^-: chloride.

CNS: central nervous system.

COS: monkey kidney cells.

Ct: cerebrocortical.

CTOP: D-Phe-Cys-Tyr-Orn-Thr-Pen-Thr-NH_2.

CTZ: chemoreceptor trigger zone.

DAG: diacylglycerol.

DAMGO: (D-Ala^2,$MePhe^4$,$gly(ol)^5$)enkephalin.

DPDPE: (D-$Pen^{2,5}$)enkephalin.

DSLET: (D-Ser^2,Leu^5,Thr^6)enkephalin.

EDTA: ethylenediamine-tetraacetic acid

EGTA: ethylene glycol-bis(β-aminoethyl ether)N,N,N,N-tetraacetic acid

FCS: foetal calf serum.

Fura-2 AM: fura-2 acetoxymethylester.

GABA: γ-aminobutyric acid.

GABA-T: GABA transaminase.

GAD: glutamic acid decarboxylase.

GC: gas chromatography.

GDH: glutamate dehydrogenase.

GI: gastrointestinal.

$_{i}$GluR: ionotropic glutamate receptors.

$_{m}$GluR: metabotropic glutamate receptors.

GP: graded potential.

HCl: hydrochloric acid.

HPLC: high performance/pressure liquid chromatography.

HTM: high threshold mechanoreceptors.

I_{Ca2+}: calcium current.

I_H: voltage dependent K^+ current.

I_K: hyperpolarising potassium current.

IP_3: inositol triphosphate.

K^+: potassium.

LC: locus coeruleus.

L-trans-PDC: L-trans pyrrolidine,2,4-dicarboxylate.

MAC: minimum alveolar concentration.

α-MEM: α-minimum essential medium.

MEM: minimum essential medium.

Mg^{2+}: magnesium.

MP: membrane potential.

Na^+: sodium.

NAD^+: nicotinamide adenine dinucleotide (oxidised form).

NADH: nicotinamide adenine dinucleotide (reduced form).

$NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidised form).

NADPH: nicotinamide adenine dinucleotide phosphate (reduced form).

NBCS: new born calf serum

NMDA: N-methyl-D-aspartate.

NO: nitric oxide.

NaOH: sodium hydroxide.

NRM: nucleus raphe magnus.

NRPG: nucleus reticularis paragigantocellularis.

NSB: non-specific binding.

PAG: periaquiductal grey.

PI: phosphoinositide.

PLC: phospholipase C.

PLD: phospholipase D.

PMN: polymodal nociceptors.

PNS: peripheral nervous system.

rac: racemic.

RAS: recticular activating system.

SG: substantia gelatinosa.

THA: DL-threo-β-hydroxyaspartate.

VSCC: voltage sensitive calcium channel.

Contents

Chapter 1. Introduction

1.1. Structure of the Central Nervous System (CNS)

The nervous system can be divided into the central nervous system (CNS), comprised of the brain lying within the skull, and the spinal cord lying within the vertebral canal and the peripheral nervous system (PNS) with fibres extending from the brain and spinal cord to all points of the body. The CNS is composed of more than 10 thousand million interconnecting neurons formed before birth that would never be replaced, but could be repaired by themselves. The brain is composed of six subdivisions: cerebrum and diencephalon forming the forebrain, midbrain, pons and medulla oblongata forming the brainstem, and the cerebellum [Vander, Sherman and Luciano 1990].

1.1.1. Forebrain

The forebrain central core is the diencephalon, surrounded by the cerebrum composed of the right and left cerebral hemispheres. The cerebral cortices form the outer shell of the cerebral hemispheres, and each cortex is divided into four lobes: frontal, parietal, occipital and temporal. The cells of the cerebral cortex are normally arranged in 6 layers within its grey matter approximately 3mm thick. The neurons within the cortex are either pyramidal, forming the major output cells of the cortex, or non-pyramidal. Deep within the cerebral hemispheres are the subcortical nuclei, including the basal ganglia that play an important role in the control of movement, posture and behaviour. The diencephalon is comprised of the thalamus, a cluster of nuclei acting as a relay for inputs to the cortex, and the hypothalamus that is crucial to homeostatic regulation and also the regulation of behaviour [Vander, Sherman and Luciano 1990].

1.1.2. Brainstem

This is the stalk of the brain through which all the nerve fibres that relay signals between the spinal cord and the cerebrum or cerebellum pass. The information transmitted between the brain stem and the cerebellum is by three large bundles of nerve fibres: cerebellar peduncles. The grey matter running through the core of the brain stem is the part of the brain essential for life, the reticular formation. It is composed of large loosely arranged

interneurons that receive and integrate information from all regions of the CNS [Vander, Sherman and Luciano 1990].

1.1.3. Cerebellum

An outer layer of cells, the cerebellar cortex and several deeper cell clusters, the cerebellar nuclei form the cerebellum. This structure is chiefly involved with skeletal muscle functions where it acts as a centre to co-ordinate and learn movements, and to control both posture and balance. To carry out these functions the cerebellum receives information from the muscles and joints, skin, eyes and ears and also from the parts of the brain involved in the control of movement [Vander, Sherman and Luciano 1990].

1.1.4. Spinal Cord

This is a cylinder of soft tissue, containing a central butterfly-shaped area of grey matter (as the structures lack myelin) composed of interneurons, cell bodies and dendrites of efferent neurons, the fibres of afferent neurons and glial cells. Around the grey matter are axons whose pathways run longitudinally through the cord. Afferent fibres enter the cord from the peripheral nerves on the dorsal side of the cord via the dorsal roots, and the dorsal root ganglia (small bumps on the dorsal roots) contain the cell bodies of the afferent neurons. The axons of efferent neurons leave the spinal cord on the ventral side via the ventral roots. The dorsal and ventral roots from the same level combine to form a pair of spinal nerves, one on each side of the spinal cord [Vander, Sherman and Luciano 1990].

1.2. Amino Acid Neurotransmitters

There are several amino acids that act throughout the CNS as neurotransmitters, including: γ-aminobutyric acid (GABA), glycine, aspartate and glutamate (Fig 1.1). GABA and glycine are inhibitory amino acids whereas glutamate and aspartate are excitatory [Nicholls 1989]. GABA occurs in abundance in brain tissue (2-10μmol/g tissue), and trace amounts are found in other mammalian tissues. It is formed from glutamic acid by the action of glutamic acid decarboxylase and metabolised by a transamination reaction to succinic semialdehyde (Fig 1.2) [Rang, Dale and Ritter 1995]. GABA is thought to function as an inhibitory transmitter in many different CNS pathways. Glycine is also considered as an inhibitory amino acid, however it does exert a modulatory role at the

NMDA receptor (see Section 1.5.1, page 8) [Kemp and Leeson 1993]. When applied iontophoretically to motorneurons or interneurons glycine produces an inhibitory hyperpolarisation indistinguishable from the inhibitory synaptic response. It is present at high concentrations within the grey matter of the spinal cord, approximately 5μmol/g tissue and is formed by the transamination of glyoxylate (Fig 1.2). Glycine also has a role in the NMDA receptor mediated release of glutamate (see Section 1.5.1, page 8). Amino acids particularly glutamate and aspartate are the principal and ubiquitous transmitters mediating fast excitatory synaptic responses in the CNS. Glutamate is widely and uniformly distributed across the CNS and has an important metabolic role in both carbohydrate and nitrogen metabolism. The metabolic and neurotransmitter pools are linked by transaminase enzymes that catalyse the interconversion of glutamate and α-oxoglutarate (Fig 1.2). The main origin of glutamate and aspartate are in the cellular amino acid pools and are important in the Krebs cycle intermediates, although glutamine also serves as a source [Hicks et al., 1987].

Excitotoxic damage to neurons and glia may develop as a consequence of excessive release of excitatory amino acids after primary impact injury, ischaemic events and haematoma as illustrated in animal studies [Inglis et al., 1990, Bullock et al., 1991A & B, Kawamata et al., 1991]. The remainder of this introduction will concentrate on glutamate, but the reader is referred to the following reviews on aspartate, glycine and GABA [McMahon and Nicholls 1991; Nicholls 1989].

Fig 1.1. Structures of the principle amino acid neurotransmitters GABA (A), glycine (B), aspartate (C) and glutamate (D).

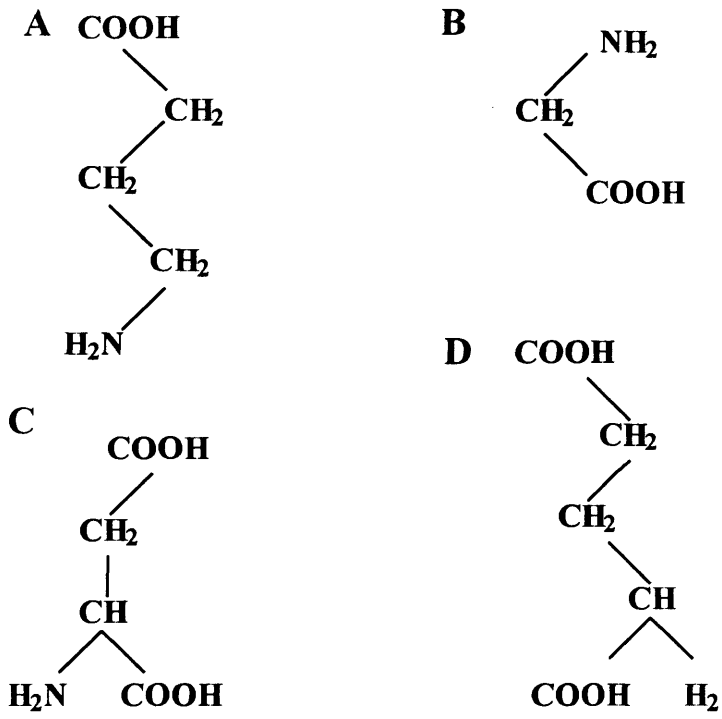
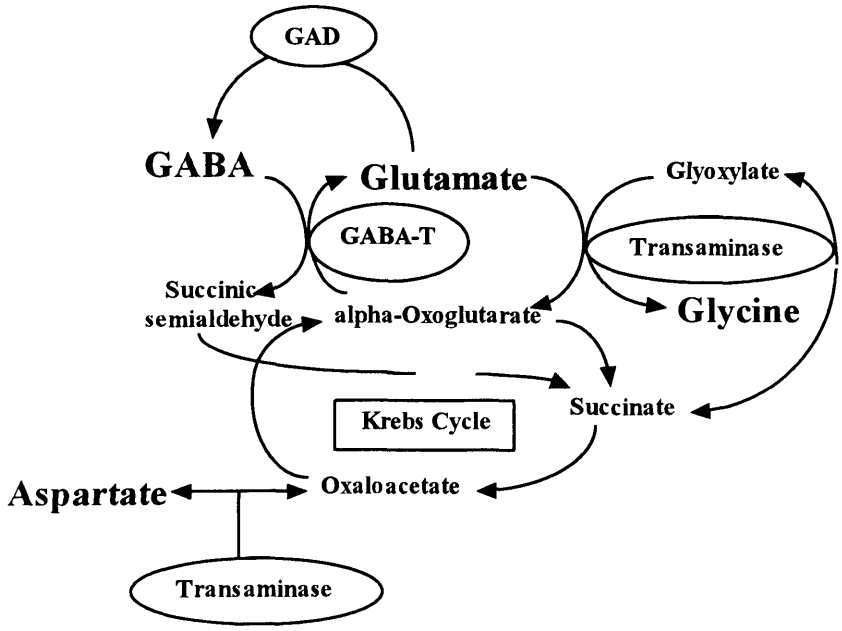


Fig 1.2. Metabolism of transmitter amino acids within the CNS. Transmitter substances are in larger typing.



Key: GAD, glutamic acid decarboxylase; GABA-T, GABA transaminase.

1.3. Glutamate

The amino acids L-glutamate and L-aspartate are widely considered to be the predominant excitatory neurotransmitters in the mammalian CNS. The most abundant free amino acid involved in a variety of reactions critical to intermediary metabolism is glutamate [Greene and Greenamyre 1996]. Glutamate is a dicarboxylic amino acid that serves in the central and peripheral nervous systems as a nonessential amino acid for general cellular metabolism and as an excitatory neurotransmitter [Rothstein 1996]. A number of glutamatergic pathways have been identified by employing a combination of experimental lesions, release and uptake studies, electrophysiology and histochemistry. These include, but are not limited to, hippocampal pathways, cerebellar afferents and parallel fibres, cortical association fibres and corticofugal fibres projecting to the striatum, thalamus, brainstem and spinal cord [Greenamyre and Porter 1994; Headley and Griller 1990]. Under resting conditions, the glutamate concentrations in the extracellular space, the presynaptic cytoplasm and the lumen of the glutamatergic synaptic vesicles are of the order of 1μM, 10mM and 100mM respectively, and these concentrations are maintained by electrogenic carriers [Nicholls and Attwell 1990] (Chapter 1, Section 1.7, page 17). Synaptic concentrations of glutamate are controlled by a balance between release and reuptake, and the failure of the electrogenic carriers can result in the accumulation of extracellular glutamate consequently leading to cell death (Chapter 1, Sections 1.6 & 1.7, pages 16 & 17).

1.3.1. Membrane Potentials

Before discussing glutamate release we must briefly cover membrane potentials (MP) and action potentials (AP). All cells under normal resting conditions have a potential difference (resting membrane potential) across their plasma membrane. In neurons the resting membrane potential is generally in the range of -40 to -75mV [Vander, Sherman and Luciano 1990]. The magnitude of the resting membrane potential is determined by the difference in ion concentrations of the intracellular and extracellular fluids and the plasma membrane permeability's to these different ion species. Na^+, Cl^- and K^+ play the most important role in the generation of the resting membrane potential (Table 1.1). Na^+ has a tendency to enter the cells along its concentration gradient. A sodium/potassium plasma membrane active transport system is responsible for pumping Na^+ out of the cell and K^+

into the cell, maintaining the resting membrane potential [Vander, Sherman and Luciano 1990].

Table 1.1. Distribution of major ions across the plasma membrane of a typical nerve cell at rest.

	Concentration (mmol/litre)	
Ion	**Extracellular**	**Intracellular**
Na^+	150	15
Cl^-	110	10
K^+	5	150

1.3.2. Graded Potentials (GP) and Action Potentials (AP)

A GP is a change in membrane potential confined to a relatively small region of the membrane and it dies out within 1-2mm from its site of origin. These are called GPs as the magnitude of the potential change is variable and is related to the magnitude of the stimulus.

APs are very different from graded potentials. They are rapid alterations in the membrane potentials that may last milliseconds during which time the membrane potential may change from -70 to +40mV, and then repolarise to its resting membrane potential. Nerve, muscle and some gland cells have plasma membranes capable of producing APs, and are called excitable membranes. AP propagation is the mechanism by which the nervous system communicates over long distances. Following a depolarising stimulus, an AP is propagated along the axon altering the membranes permeability's to Na^+ and K^+. Na^+ channels open, and Na^+ rushes into the cell reversing its polarity. The movement of Na^+ ions in, results in a net charge closing Na^+ and opening K^+ channels causing repolarisation. Not all depolarisation's trigger the positive feedback relationship that leads to an AP. An AP will only occur when the net movement of positive ions is inward. The 'threshold' is the membrane potential at which the predominant movement of ions across the membrane first changes from outward to inward. Stimulation of an AP of more than the threshold

potential does not produce a AP of greater amplitude. APs either occur maximally or not at all, they are 'all-or-none' [Vander, Sherman and Luciano 1990].

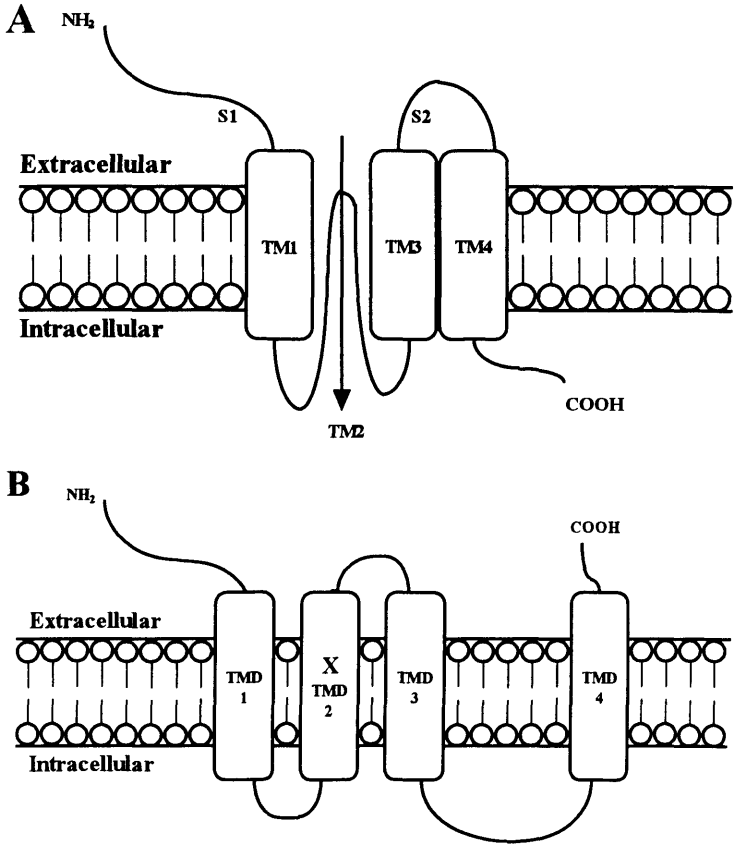
1.4. Glutamate Release

The change in membrane potential achieved by the propagation of an AP along the axon, activates voltage sensitive calcium channels (VSCCs) allowing the rapid movement of Ca^{2+} into the cell, producing a rapid transient rise in intracellular Ca^{2+}, $[Ca^{2+}]_i$ [Adam-Vizi 1992]. Any neurotransmitter present in the presynaptic terminal is stored in membrane-bound synaptic vesicles. The synaptic vesicles fuse with specific vesicle-attachment sites (clathrin-associated adaptor proteins) on the inner surface of the presynaptic plasma membrane [Traub 1997]. The fusion of the vesicles weakens the presynaptic plasma membrane resulting in the exocytotic release of vesicular glutamate from pre-synaptic terminals into the synaptic cleft.

1.5. Glutamate Receptors

The released glutamate from vesicles in the presynaptic terminal passes across the synaptic cleft where it can bind to the glutamate receptors located on the post-synaptic terminal and simultaneously feed-back onto metabotropic and NMDA glutamate receptors located on the presynaptic terminal [Sanchez-Prieto et al., 1996; DeBiasi et al., 1996; Conti et al., 1997]. To date glutamate receptors have been classified into four major subtypes: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-ethyl-4-isoxazole proprionic acid (AMPA), kainate and metabotropic glutamate receptors. The first three receptors are ionophores in the cell membrane (see Fig 1.3), and the latter is a seven membrane spanning domain linked to a G-protein that modulates the production of intracellular messengers [Sheng 1997].

Fig 1.3. Schematic representation of the transmembrane domain (TMD) topology of ionotropic glutamate receptors. A represents a NMDA receptor and S1 and S2 are sites associated with ligand binding [adapted from Small and Buchan 1997]. B represents an AMPA/kainate receptor and X indicates the Q/R site (see text for explanation).



1.5.1. NMDA Receptors

The NMDA receptors are heteromeric pentamers which form ligand-gated ion channels [Behe et al., 1995] (Figs 1.3 & 1.4). The subunits are products of two gene families; the NR1 gene which undergoes alternative splicing to yield 8 different products and the four NR2 genes: A, B, C and D (see Fig 1.5) [Lambolez et al., 1992; Monyer et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Ikeda et al., 1992; Ishii et al., 1993]. The predicted molecular weights of the gene products are between 103-163KDa, comprising of between 920-1456 amino acids. Within the NR2 gene family there is approximately 40-50% homology, but between NR1 and NR2 as little as 20% homology [Mishina et al., 1993]. NR1 is found ubiquitously throughout the mammalian brain, and every NMDA receptor contains at least one NR1 subunit with one or more NR2 subunits. NR2 subunits are not uniformly expressed throughout the brain: NR2A is found in the cortex, hippocampus and cerebellum, NR2B is found in the cortex and hippocampus, NR2C is

only found in the cerebellum and NR2D is only found in the olfactory bulb [Buller et al., 1994].

The NMDA receptor channel complex has an integral channel that is highly permeable to Ca^{2+} (5 times more permeant than any other ionotropic glutamate receptor), Na^{+} and K^{+}. Due to the narrow pore of the channel, (a mean diameter of 5.5Å) the passage of larger cations is restricted [Villarroel et al., 1995]. Physiological concentrations of extracellular Mg^{2+} block the NMDA receptor in a voltage dependent manner, relieved at depolarisation beyond -30mV [Small and Buchan 1997]. The sensitivity of the Mg^{2+} block of NMDA receptors is dependent upon the activity of the intracellular kinases and the NMDA receptor subunit combinations. Zn^{2+} also interacts with the NMDA receptor, at a different site to Mg^{2+}. At submicromolar concentrations of Mg^{2+} a potentiation of the NMDA response is seen [Hollmann et al., 1993], a voltage-independent block is achieved at micromolar concentrations [Westbrook and Mayer 1987], and with greater than micromolar concentrations a partial block is observed [Hollmann et al., 1993]. As with Mg^{2+} the effects of Zn^{2+} are subunit specific.

Polyamines can also interfere with NMDA responses depending on the concentration and the NMDA subunit involved. At higher concentrations polyamines get physically stuck in the permeation pathway of the channel causing a partial block [Westbrook and Mayer 1987], and at low micromolar concentrations polyamines potentiate the response [McGurk et al., 1990]. Glycine serves as a co-agonist at the NMDA receptor, with an affinity in the nanomolar range, by interacting with a distinct extracellular site. The physiological concentration of glycine lies in the micromolar range, therefore given its affinity the NMDA receptor usually exists in the 'glycine-primed' state [Curtis and Johnson 1974; Johnson and Asher 1990].

There are many modulatory sites on the NMDA receptor other than the glycine and polyamine sites. These include the binding of competitive antagonists to the NMDA/glutamate binding site reversible by the application of glutamate/NMDA, and the binding of non-competitive antagonists to sites within the channel pore (see Table 1.2) [Small and Buchan 1997].

Fig 1.4. The structure of the activated multifunctional NMDA receptor channel complex.

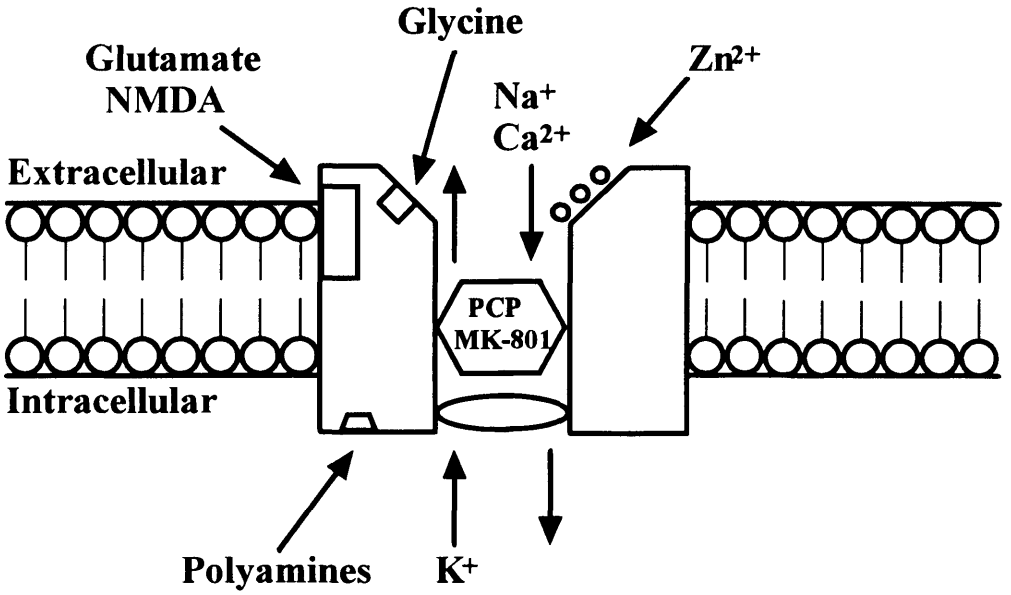


Table 1.2. Different classes of synthetic NMDA receptor ligands, and their sites of action.

Class (Site of Action)	Compounds
Competitive (NMDA/glutamate site)	D-AP_5, AP_7.
Non-Competitive (High Affinity) (Within the channel pore)	MK-801/dizocilpine, phencyclidine.
Non-Competitive (Intermediate Affinity) (Within the channel pore)	Dextrometorphan.
Non-Competitive (Low Affinity) (Within the channel pore)	Ketamine, kyurenate, amantidine, magnesium.
Glycine Site	Felbamate.
Partial Agonist (NMDA/glutamate site)	HA-966.
Polyamine Site	Ifenprodil

Key: AP_5, 2-amino-5-phosphonobutyrate; AP_7, 2-amino-7-phosphonobutyrate.

1.5.2. AMPA Receptors

The AMPA and kainate subclasses of the non-NMDA receptor have been defined by ligand binding studies, where AMPA binds with a higher affinity than kainate to AMPA

receptors and vice versa at kainate receptors [Lees 1996]. Following the cloning of the first glutamate receptor subunit (GluR1/A) by Hollmann and co-workers in 1989, GluR2/B, 3/C and 4/D were soon isolated [Keinanen et al., 1990; Boulter et al., 1990], all showing high affinities towards AMPA (see Fig 1.5). The subunits are approximately 900 amino acids in length, sharing ~70% amino acid identity and occur in two major forms [Sommer et al., 1990]. The two forms are named 'flip' and 'flop' [Tolle et al., 1993]. The prenatal brain expresses mostly 'flip' and these forms persists through life, whereas the 'flop' appears from early postnatal onwards and is co-expressed with 'flip' in many cells [Seeburg 1993].

GluR2/B differs from the other three subunits as it contains an arginine residue within the Q/R site in TMII segment whereas the other three carry a glutamine residue at the same position. Channels formed from GluR1/A, GluR3/C and GluR4/D show high Ca^{2+} [Hollmann et al., 1991], Mg^{2+} and Ba^{2+} permeability [Burnashev et al., 1992; Dingledine et al., 1992]. Heteromeric AMPA receptor channels (GluR1/A/GluR2/B) show properties of a linear current-voltage relationship and a low divalent ion permeability [Jonas and Sakmann 1992]. Therefore the difference in GluR2/B dominantly determines the channel conductance and Ca^{2+} permeation [Nakanishi 1992].

GluR1/A-GluR4/D mRNAs are widely distributed in adult rat brain, particularly enriched in cortex, hippocampus and cerebellum [Hollmann et al., 1989; Keinanen et al., 1990; Boulter et al., 1990]. In cortex, GluR1/A, GluR3/C and GluR4/D differ among the layers, while GluR2/B is uniformly distributed. In the hippocampus, GluR1/A, GluR2/B and GluR3/C mRNAs are found in the dentate gyrus and in the CA1 and 3 regions, while GluR4/D mRNA is primarily located in CA1 and dentate gyrus. The lack of GluR1/A and GluR3/C in granule, and GluR2/B and GluR4/D Bergmann glia cells of the cerebellum is the area where the most marked difference occurs [Jorgensen et al., 1995].

1.5.3. Kainate Receptors

The cloning of kainate receptor subunits was achieved using low-stringency hybridisation screening with GluR1/A-4/D cDNA probes, resulting in low affinity (GluR5, GluR6 and GluR7) and high affinity (KA-1 and KA-2) receptors (see Fig 1.5) [Bettler et al., 1990;

Egeberg et al., 1991; Bettler et al., 1992; Werner et al., 1991; Herb et al., 1992]. GluR5-7 share 75-80% amino acid sequence identity with each other, but only ~40% with GluR1/A-GluR4/D, whereas KA-1/KA-2 share 70% homology with each other and only ~37% with GluR1/A-GluR4/D. Kainate receptors are widely expressed in rat brain including layer 1 and the inner laminae of the neocortex and cingulate cortex, caudate putamen, the CA3 region of the hippocampus, the reticular thalamus, the hypothalamic median eminence and the cerebellar granule cell layer, indicating that kainate receptors may be involved in all central neuronal circuits of the brain [Seeburg 1993].

GluR5 and GluR6 occur in two forms with respect to the amino acid residue occupying the Q/R (glutamine to arginine) site in TMII [Sommer et al., 1991]. GluR6 also has two additional positions in TMI modified by RNA editing (isoleucine to valine and tyrosine to cysteine). These forms occur at different frequencies in the CNS [Kohler et al., 1993]. GluR6 fully edited in TMI and TMII amounts to 65%, the un-edited GluR6 10%, and all other GluR6 forms constitutes the remaining 25% in rat brain. Only when the TMI position is edited on the GluR6 subunit does editing of the Q/R site on the TMII segment influence Ca^{2+} permeability (see Fig 1.3)

1.5.4. Metabotropic Receptors

To date eight metabotropic glutamate receptor subtypes (mGlu) have been cloned, termed $mGluR_1$-$mGluR_8$ (see Fig 1.5) [Pin and Duvoisin 1995], possessing seven membrane segments common to other G-protein coupled receptors. As the amino acid sequence identity varies between mGlu receptor subtypes, they have been grouped into three classes [Nakanishi 1992; Riedel 1996]. Within a class there is approximately 70% homology, whereas between classes this homology decreases to between 40-45%. Each class demonstrates different transduction mechanisms. Initially all mGlu receptors were thought to couple exclusively to inositol triphosphate (IP_3)/Ca^{2+} signal transduction [Schoepp et al., 1990], but this now appears to be untrue [Tanabe et al., 1992 & 1993].

$mGluR_1$ (splice variants: a, b, c, d, e) and $mGluR_5$ (splice variants: a, b) are in class I, these receptors stimulate phospholipase C by an increase in phosphoinositide turnover, and the release of Ca^{2+} from internal stores [Houamed et al., 1991; Masu et al., 1991].

Activation of class I of mGlu receptors also leads to the formation of diacylglycerol (DAG) which then activates protein kinase C. A negative coupling to K^+ channels has been reported in several neuronal and non-neuronal preparations [Baskeys et al., 1990; Charpak et al., 1990; Glaum and Miller 1992; Ikeda et al., 1995]. $mGluR_{1a}$ appears to be localised at the periphery of the postsynaptic membrane, in contrast to ionotropic Glu receptors that occupy the core of the synapse [Nusser et al., 1994]. $mGluR_{1a}$ is expected to be activated by excessive amounts of glutamate released during synaptic hyperactivity [Nicoletti et al., 1996]. In addition an unknown mGlu receptor that is said to couple to PI hydrolysis via phospholipase D and is located at the presynaptic level activation of this receptor enhances glutamate release in the presence of arachidonic acid [Herrero et al., 1992] (see Table 1.3 and Fig 1.6).

Class II consists of $mGluR_2$ and $mGluR_3$, and class III consists of $mGluR_4$, $mGluR_6$, $mGluR_7$, and $mGluR_8$ respectively, and are both negatively coupled to adenylate cyclase reducing the amount of cAMP [Tanabe et al., 1992 & 1993]. The differences between classes II and III are in their pharmacological profile for specific agonists. These receptors are also negatively coupled to voltage operated calcium channels (VOCC) [Ikeda et al., 1995; Trombley and Westbrook 1992; Chavis et al., 1994]. $mGluR_2$, $mGluR_4$ and $mGluR_7$ have been shown to be localised presynaptically [Pin and Duvoisin 1995] and pharmacological activation of all subtypes of classes II and III reduce glutamate release, the exception being $mGlu_2$ receptor antagonists which amplify the elicited release of glutamate [Di Iorio et al., 1996]. It can generally be assumed that activation of class I mGlu receptors increases neuronal excitation and excitability, whereas activation of class II or III (excluding $mGlu_2$ receptors) reduces synaptic excitation [Nicoletti et al., 1996] (Fig 1.6 and Table 1.3).

Fig 1.5. Summary of the cloned glutamate receptor subunits [Jorgensen et al., 1995].

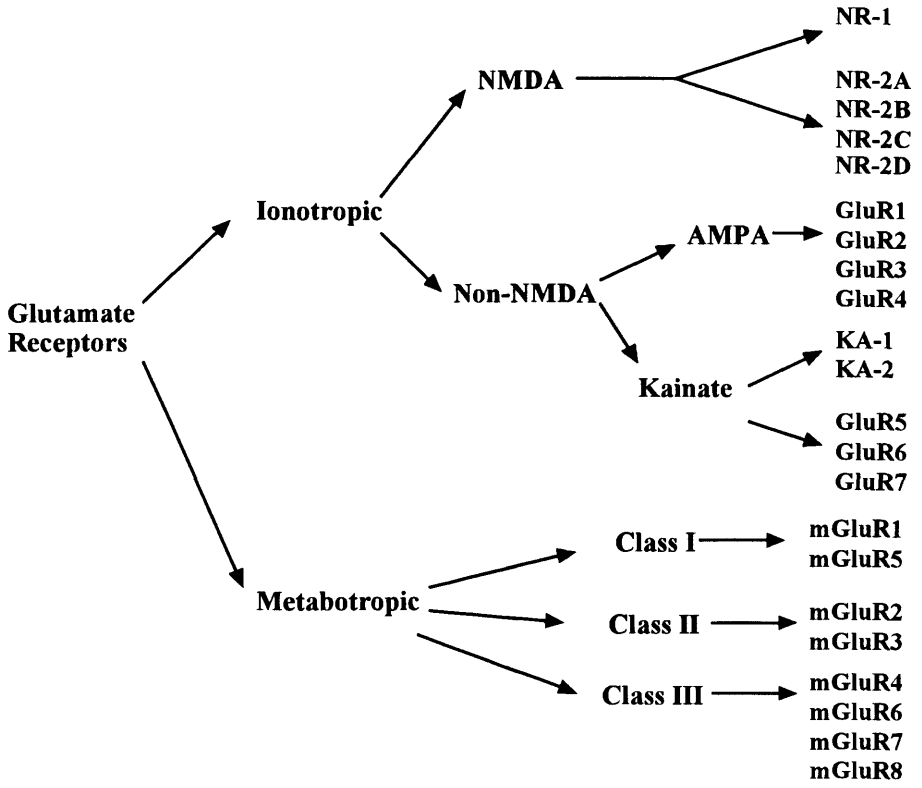
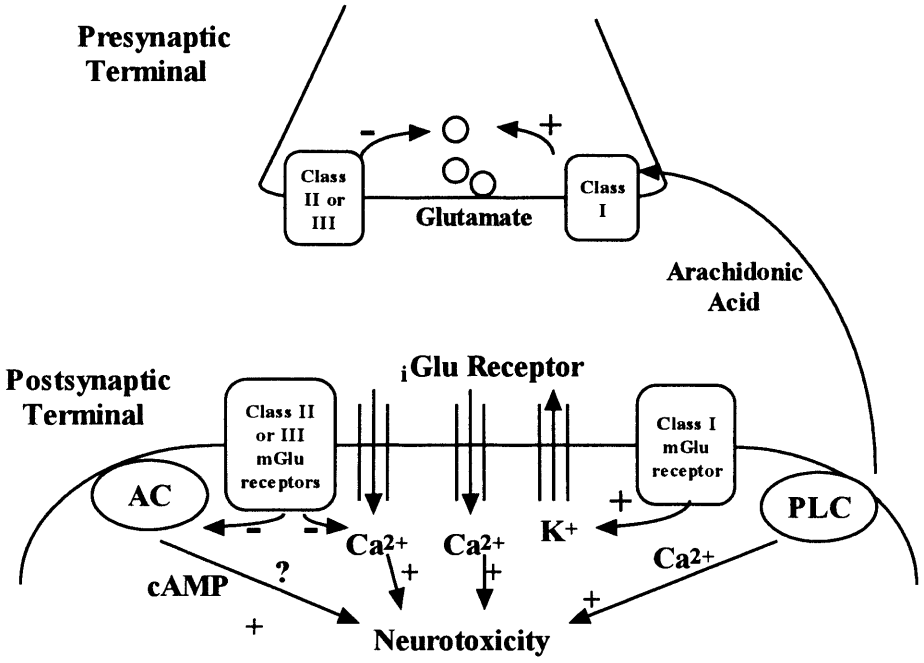


Table 1.3. Metabotropic glutamate (mGlu) receptors: pharmacology and putative role in neuronal toxicity.

mGlu receptor subtypes	Transduction Pathways	Putative Role in NeuroDegeneration	Mechanisms
Class I	↑PI, ↓I_K.	↑ Neuronal Toxicity	↑NMDA current ↑Glu Release Depolarisation and open VOCC
Class II	↓cAMP, ↓I_{Ca2+} (L, N, P-VSCC), ↑I_K.	Neuroprotection	↓cAMP ↓Ica ↓Glu Release
Class III	↓cAMP, ↓I_{Ca2+} (N, P-VOCC).	Neuroprotection?	↓Glu Release
Unknown	↑PLD		

Key: PI, phosphoinositide; I_K, potassium channels; I_{Ca2+}, calcium channels; VSCC, voltage sensitive calcium channels; PLD, phospholipase D; ↓, increase; ↑, decrease.

Fig 1.6. Schematic representation of the role of mGLU-receptor subtypes on glutamate release and the induction and progression of excitotoxic damage.



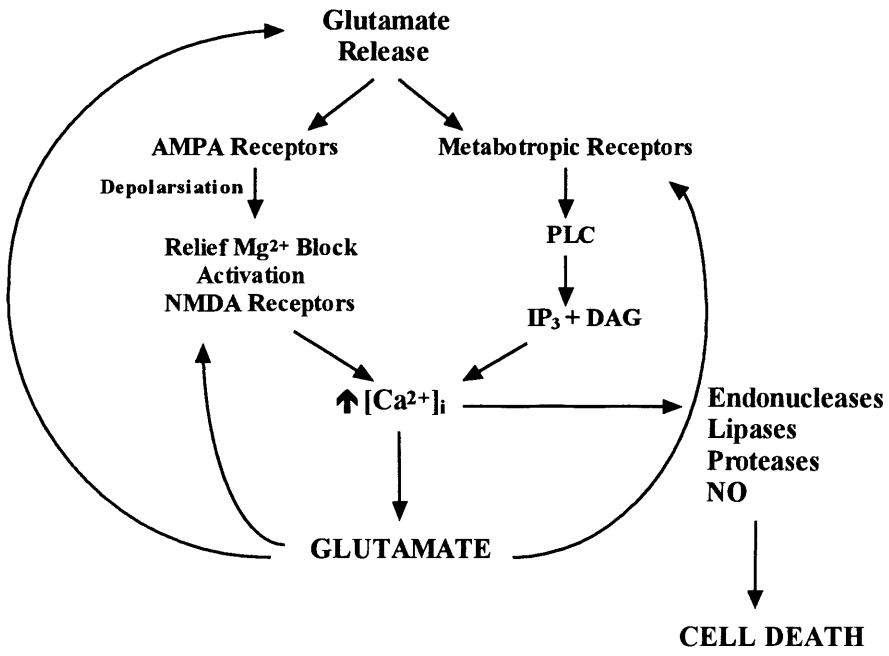
Key: PLC, phospholipase C; AC, adenylate cyclase; K^+, potassium; Ca^{2+}, calcium; iGlu receptors, ionotropic glutamate receptors; mGlu receptors, metabotropic glutamate receptors

1.6. Glutamate Excitotoxicity

During normal synaptic activity glutamate released into the synaptic cleft activates AMPA receptors but not NMDA, due to the Mg^{2+} block of the channel [Sanchez-Prieto et al., 1996]. However, when neurons are depolarised, the Mg^{2+} block of NMDA receptors is removed, and an increase in $[Ca^{2+}]_i$ is observed. Postsynaptic activation of NMDA and AMPA receptors increases $[Ca^{2+}]_i$ directly, whereas activation of postsynaptic class I metabotropic receptors results in the hydrolysis of PLC. Hydrolysis of PLC produces DAG and IP_3, the later resulting in the increase of $[Ca^{2+}]_i$. Activation of postsynaptic class II and III metabotropic glutamate receptors inhibits adenylate cyclase, decreasing cAMP and $[Ca^{2+}]_i$, attenuating neuronal toxicity [Nicoletti et al., 1996]. Following glutamate release, it can also feed back onto glutamate autoreceptors located on the presynaptic terminal, this causes a positive or negative feedback onto glutamate release [Sanchez-Prieto et al., 1996;

DeBiasi et al., 1996; Conti et al., 1997]. Presynaptic class II and III inhibit, whereas class I enhances glutamate release [Nicoletti et al., 1996]. Glutamate is normally taken back into the pre-synaptic nerve terminals or glia by acidic Na^+-dependent uptake transporters following release. Failure of the energy dependent transporters results in accumulation of high synaptic concentrations of glutamate, overactivation of the glutamate receptors, and consequently cell death [Nicholls and Attwell 1990]. The accumulation of $[Ca^{2+}]_i$, and the consequent activation of lipases and nitric oxide (NO) can result in cell death (see Fig 1.7) [Small and Buchan 1997]. All the mechanisms mentioned above contribute to the excitotoxic glutamate cascade, and have been implicated in epilepsy, brain ischaemia and amyotrophic lateral sclerosis [Rothstein et al., 1993].

Fig 1.7. Schematic diagram of the possible mechanisms involved in glutamate neurotoxicity.



1.6.1. Head Injury/Trauma (a clinical condition that may lead to excitotoxic damage)

During a traumatic event many injuries can be sustained, and a head injury has one of the most devastating outcomes. Head injuries are the most important cause of death for young adults in the USA [Goldstein 1990]. Even though there have been major advances in the management of head injury, neither the mortality rate nor the incidence of preventable

brain damage at post-mortem have decreased more than 5-10% [Bullock & Teasdale 1990]. This may be because the pathophysiological processes that damage the brain (both during and after severe head injury) have been uninfluenced by conventional therapy or the experimental drugs that have been tried [Bullock & Fujisawa 1992]. Clinical and neuropathological studies have suggested that secondary processes are responsible for the substantial part of the brain damage, as 30-40% of patients who die after severe head injury have spoken at some stage after the impact [Blumberg et al., 1989]. Several animal studies have suggested that excitotoxic damage to neurons and glia may develop as a consequence of excessive release of excitatory amino acids after primary impact injury, ischaemic events and haematoma [Inglis et al., 1990; Bullock et al., 1991A & B; Kawamata et al., 1991]. Based on this information, the management of head injuries should not only include glutamate antagonists, but also analgesic and sedative agents that may inhibit the release of excitatory amino acids. These could possibly limit any further neuronal damage by either inhibiting glutamate release or interfering with the glutamate cascade leading to neurotoxicity.

1.7. Glutamate Uptake Transporters

The general properties of plasma-membrane amino acid transport systems that emerged in the 1980s were; to exhibit Michaelis-Menten kinetics, to lead to the accumulation of amino acids within the cell at concentrations of 3-4 orders of magnitude higher than that outside, to utilise energy, and to require Na^+ [Erecinska 1987]. The driving force for amino acid transport was postulated to be the downhill movement of Na^+ into, and K^+ out of the cell by Na^+/K^+ ATPase. To date four distinct sequences for glutamate transporters have been published (Fig 1.8).

1.7.1. GLT-1 Transporter

Pines et al., 1992, used an antibody that blocked glial glutamate transport to screen a rat brain cDNA library. They succeeded in isolating and expressing a glutamate transporter of 573 amino acids with 8 predicted transmembrane regions, termed GLT-1 (see Fig 1.8). This transport system was reconstituted in HeLa cells and was reported to take up Na^+ and L-glutamate, and extrude K^+ in an electrogenic process. The transport of [^{3}H]L-glutamate was inhibited by L-glutamate (i.e. by isotope dilution) but not D-glutamate, and was Na^+

dependent. Other neurotransmitters did not interact with GLT-1 as there was no inhibition of [^{3}H]L-glutamate uptake with unlabelled GABA, dopamine, noradrenaline and serotonin. The apparent K_m was ~2μM. Also cloned from mouse cerebellum was a cDNA encoding a protein of 572 amino acids, sharing ~93% amino acid identity with GLT-1, termed MGLT1 [Mukainaka et al., 1995].

1.7.2. EAAC1 Transporter

The next transporter cloned was by Kanai and Hediger 1992. They isolated a clone from rabbit small intestine using expression in Xenopus oocytes, that possessed 524 amino acids, 57Kda in molecular mass and 10 transmembrane regions, it was named EAAC1 (see Fig 1.8). EAAC1 transcripts were found in specific neuronal structures in the CNS as well as in the small intestine, kidney, liver and heart. When EAAC1 was expressed in Xenopus oocyes, it induced [^{14}C]L-glutamate uptake to 1,300-fold above that of water-injected controls. The uptake of [^{14}C]L-glutamate was inhibited by L- and D-aspartate but not D-glutamate, while the uptake of [^{14}C]L-aspartate was inhibited by both D-aspartate and L-glutamate but not D-glutamate. The transport of L-glutamate, L- and D-aspartate by EAAC1 had apparent K_m's of 12.2, 6.5 and 7.5μM. The uptake was Na^+- but not Cl^--dependent.

EAAC1 has been isolated from several species (see Fig 1.8). Kanai et al., 1995, isolated EAAC1 cDNA from rat cerebral cortex. They revealed expression of EAAC1 also in the GABAergic Purkinje cells suggesting that it serves to provide these neurons with glutamate as a precursor for GABA synthesis. Another subtype was cloned from mouse cerebellum. A cDNA of 523 amino acids, sharing ~89% amino acid identity with the rabbit EAAC1, termed MEAAC1 has also been identified [Mukainaka et al., 1995]. Bjoras et al., 1996 isolated the cDNA for another rat brain glutamate transporter (REAAC1) encoding a sodium dependent glutamate transporter with similar substrate selectivity as for EAAC1 and possessing 90 and 97% amino acid identity to the rabbit EAAC1 and the mouse EAAC1 respectively.

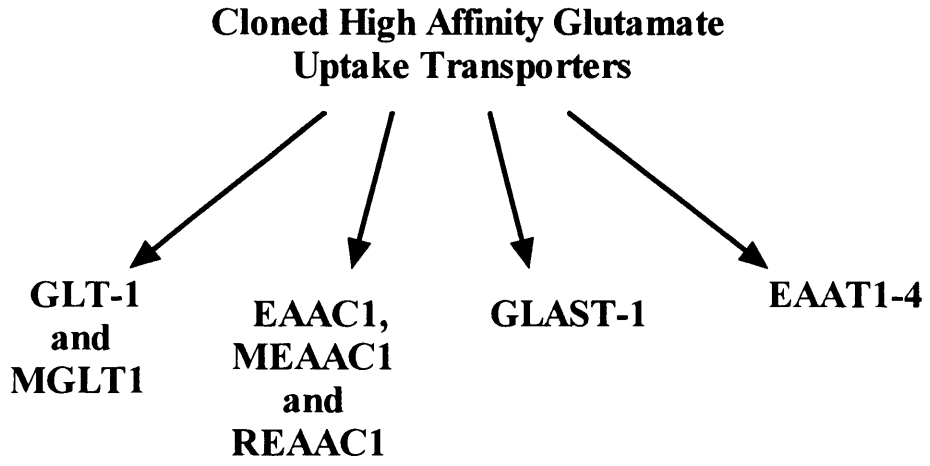
1.7.3. GLAST-1 Transporter

The third transporter was isolated from a rat brain cDNA library by Storck et al., 1992. It encoded a protein of 543 amino acids, predicted a 6 transmembrane domain structure on hydrophobicity analysis, was specifically expressed in brain and termed GLAST-1 (see Fig 1.8). The amino acid transport properties of GLAST-1 were investigated using the Xenopus oocyte expression system. The transporter is highly specific for L-glutamate and L-aspartate, but not L-alanine, L-leucine, L-glutamine, L-arginine and L-methionine. Transporter function is strictly dependent on Na^+ and the K_m values determined for L-glutamate and L-aspartate were ~77 and 65μM. Although GLAST was widely distributed throughout the cerebrum, slightly higher levels were found in the hippocampus. Within the cerebellum GLAST-1 was specifically expressed in the stratum gangliosum made up primarily by Purkinje and Bergmann glia cells. Even though Purkinje cells do not use glutamate and aspartate as neurotransmitters, they do receive excitatory signals from climbing and parallel fibres.

1.7.4. EAAT1-4 Transporters

Based on the original rabbit EAAC1 transporter, cloned by Kanai and Hediger 1992, a group of human amino acid transporters (EAAT1-3) [Arriza et al., 1994], were cloned which did not account for all the pharmacologically distinguishable glutamate uptake activities (see Fig 1.8). Each EAAT subtype was found in all human brain regions, but the expression of EAAT1 predominated in the cerebellum. EAAT2 mRNA was largely restricted to the brain, whereas EAAT1 and EAAT3 were also expressed in various non-neronal tissue. The affinities of the EAAT subtypes for L-glutamate were similar, with K_m varying from 48-97μM in a mammalian cell assay and from 18-28μM in oocytes. Fairman et al., 1996 identified an additional member of the EAAT family expressed predominantly in the cerebellum (see Fig 1.8). Identified were two independent full length cDNAs, encoding EAAT4. The amino acid sequence predicted by the EAAT4 did not correspond to the any of the glutamate transporters already cloned. EAAT4 has high affinity for L-aspartate and L-glutamate and in Xenopus oocytes expressing EAAT4, both L-aspartate and L-glutamate elicited a current predominantly carried by Cl^-.

Fig 1.8. Flow chart of the cloned high affinity glutamate uptake transporters.



1.8. Opioids

Opium (from the Greek word for juice) is the dried exudate from unripe seed capsules of the opium poppy, and has been used for many centuries for the relief of pain. There are over 20 alkaloid constituents present in opium and in 1806 one of the active constituents of opium was identified as morphine after which its medicinal use became world-wide. The molecular effects of morphine were described much later in 1973, where stereospecific opioid binding sites within the central nervous system were reported [Pert and Snyder 1973; Terenius 1973]. Pert and Snyder in 1973 reported that opiate receptor binding of [^{3}H]naloxone was confined to the brain of rats, guinea-pigs and mice, as no binding was found in the small intestine of the guinea-pig, human erythrocytes, baker's yeast and rat liver. The binding in the corpus striatum was four times that in the cerebral cortex, and no opiate receptor binding was found in the cerebellum. They found that the analgesic activity of opiates was highly stereospecific, as the activity resided in the D(-)-morphine isomer. Morphine is metabolised in the liver and also in the microsomal fraction of the CNS [Wahlstrom et al., 1988 & 1989] forming morphine-3-glucuronide and morphine-6-glucuronide. The analgesic potency of micro-injections of morphine-6-glucuronide into the periaqueductal grey (an area in the mid brain, known to be associated with the descending inhibitory control of pain) has reported to be 20-fold more potent than that of morphine [Pasternak et al., 1987].

1.8.1. Pain

The theory of pain was described by Descartes in 1644, who believed that the administration of damaging stimuli to the skin led to the activation of the cerebral cortex. This was classed as a 'Specificity Theory' as it was thought that the noxious information was transferred directly to the brain with no opportunity for modulation by the activity of other pathways. In 1965 Melzack and Wall formulated a model which incorporated the concept of multi-modal interaction at the dorsal horn level of the spinal cord. This 'Gate Theory' proposed that activity in non-nociceptive afferents was capable of influencing the intensity of the 'pain message'. To date the physiology of pain transmission is such that elements of both theories co-exist. Pain has been described as a subjective experience, interpreted as symptomatic evidence of impending or actual tissue damage [Bowman and Rand 1990]. It has also been associated with electrical activity in small diameter primary afferent fibres of peripheral nerves. The 'quality' of the pain associated with different types of tissue damage varies. The location, intensity and 'quality' of the pain depends upon the pattern of sensory input from a variety of nociceptors and low threshold receptors. Nociception is the mechanism whereby noxious peripheral stimuli are transmitted to the CNS. Pain is a subjective experience, not always associated with nociception.

1.8.1.1. Nociceptive Afferent Neurons

Nociceptive afferent nerves are distinguished from other sorts of mechanical and thermal receptors by their high threshold, as they are normally activated by stimuli sufficiently intense to cause tissue damage. There are two types of afferent fibres associated with nociception: fine myelinated Aδ fibres (also called high threshold mechanoreceptors-HTM) and non-myelinated C fibres (also called polymodal nociceptors-PMN) (Table 1.4).

Table 1.4. Summary of the sensory modalities transmitted by peripheral fibres in primates.

	Aδ-fibres	C-fibres
Diameter (μm)	0.5-6	0.2-2.6
Conduction Velocity (m/sec)	5-30	0.4-1.3
Nociceptors	55%	90%

1.8.1.2. Central Processing of Nociceptive Information

The cell bodies of spinal nociceptive afferent fibres lie in dorsal root ganglia; fibres enter the spinal cord via the dorsal roots and end in the grey matter of the dorsal horn. Nociceptive fibres make synaptic contact with laminae I and II neurons, and much attention has focused on the function of lamina II (the substantia gelatinosa-SG) the cells of which form a network of short projections to laminae I and V cells of the same segment. The SG neurons exert a predominantly inhibitory effect on synapses between the primary afferent and the spinothalamic tract transmission neurons. They can interrupt transmission at the first synapse of the pain pathway, giving rise to the term 'gate theory' [Melzack and Wall 1965]. The SG is also rich in endogenous opioid peptides (see Section 1.8.2) and opioid receptors (see Section 1.8.3). Based on this theory, SG cells respond both to the activity of afferent fibres entering the cord and to the activity of descending pathways (see Section 1.8.1.3). Further studies by Fields and Basbaum 1989, illustrate that similar 'gate' mechanisms also operate in the thalamus.

1.8.1.3. Descending Inhibitory Controls

Descending pathways are one of the gating mechanisms controlling impulse transmission in the dorsal horn. The periaquiductal grey (PAG) area of the midbrain is a key part of this descending system. Reynolds in 1969 found that electrical stimulation of this area in the rat caused intense analgesia (specific to nociceptive stimuli) such that abdominal surgery could be performed without anaesthesia . The PAG receives inputs from other brain regions, including the hypothalamus, cortex and thalamus and this area might be responsible for the mechanism where inputs act to control the 'gate' in the dorsal horn.

The nucleus raphe magnus (NRM) is the main neuronal pathway activated first by PAG stimulation, forming synaptic connections on dorsal horn interneurons. The major transmitter is 5-HT, and the interneurons act to inhibit the discharge of spinothalamic neurons. The NRM also receives an input directly from the spinothalamic neurons via the nucleus reticularis paragigantocellularis (NRPG). Therefore the descending inhibitory system may form part of a regulatory feedback loop whereby transmission through the dorsal horn is controlled by the amount of activity reaching the thalamus. The locus coeruleus (LC) is considered the main noradrenergic nucleus involved in the descending control of pain. Its main inputs are from the NRPG but does not receive a direct input from the PAG. LC stimulation releases noradrenaline, producing a similar inhibitory effect on transmission in the dorsal horn [Stamford 1995].

1.8.1.4. Neurotransmitters in the Nociceptive Pathway

Fast synaptic transmission at the first synapse in the dorsal horn is produced by the release of glutamate from primary afferent neurons acting on AMPA receptors, whereas the slower response is mediated by NMDA receptor activation. The activation of NMDA receptors has recently been shown to cause a release of substance P from small diameter primary sensory afferent pain fibres. The pain and the morphological changes that occurred upon the activation of NMDA receptors were significantly reduced by substance P-receptor antagonists or by the elimination of substance P-containing primary afferent fibres [Liu et al., 1997]. The PAG is rich in [Met]-enkephalin and β-endorphin, and [Met]-enkephalin is also found in the NRM and in superficial layers of the dorsal horn (SG). The application of opioid antagonists can prevent electrically induced analgesia in these areas, therefore opioid peptides may function as transmitters in this system. 5-HT is the transmitter of inhibitory neurons from the NRM to the dorsal horn (NRPG) and noradrenaline is the transmitter from the LC to the dorsal horn [Stamford 1995].

1.8.2. Endogenous Opioid Ligands

An endogenous substance in the brain acting as an agonist at opiate receptor sites was described by Terenius and Whalstrom 1975; Hughes 1975A. This same substance was characterised later in the same year by Hughes et al., 1975B, as a low molecular weight

peptide termed enkephalin. Further studies by Hughes et al., 1975A identified [Met^5]- and [Leu^5]-enkephalin (Fig 1.9).

Bradbury et al., 1976, using a pig pituitary gland isolated β-lipotropin (hormone), C-fragment (residues 61-91 of β-lipotropin), C'-fragment (61-87), β-melanotropin (41-58) and N-fragment. Using these peptides their ability to reduce the binding of [^{3}H]dihydromorphine and [^{3}H]naloxone to a membrane preparation from the rat brain was examined. All the peptides were able to inhibit the binding of [^{3}H]dihydromorphine and [^{3}H]naloxone to brain opiate receptors, with the active peptides all containing the N-terminal sequence Tyr-Gly-Gly-Phe. Interestingly, the C-fragment was found to have the highest affinity for the displacement of both [^{3}H]dihydromorphine and [^{3}H]naloxone, even greater than that of Met-enkephalin, and was termed β-endorphin (Fig 1.9).

Goldstein et al., 1979 described the opioid property of dynorphin, the sequence of which corresponded to the terminal sequence of a novel porcine pituitary endorphin. It also contained [Leu]-enkephalin in its amino acid sequence (Fig 1.9). In the guinea-pig ileum it was found to be 700 times more potent than [Leu]-enkephalin, the effects of which were completely blocked by naloxone. However the concentration of naloxone needed to block its effects were 13× that needed to block the effects of [Leu]-enkephalin or normorphine.

More than 20years after the discovery of enkephalin, Zadina et al., 1997 reported the discovery of two potent and selective endogenous agonists for the μ-opiate receptor, endomorphin-1 and endomorphin-2. Endomorphin-1 (see Fig 1.9) is reported to have a high affinity (K_i = 360pM) and selectivity (4,000- and 15,000-fold preference over δ and κ receptors) for the μ receptor. It is reported to be more effective than the μ selective analogue DAMGO at the μ receptor in vitro and produced potent and prolonged analgesia in rats.

Fig 1.9. The amino acid sequences of the pentapeptides [Met^5]- and [Leu^5]-enkephalin, β-endorphin, dynorphin and endomorphin-1 and -2.

[Met^5]-enkephalin H-Tyr-Gly-Gly-Phe-Met-OH

[Leu^5]-enkephalin H-Tyr-Gly-Gly-Phe-Leu-OH

β-endorphin H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Val-His-Lys-Lys-Gly-Gln-OH

dynorphin H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH

endomorphin-1 H-Tyr-Pro-Trp-Phe-NH_2

endomorphin-2 H-Tyr-Pro-Phe-Phe-NH_2

1.8.3. Opioid Receptor Subtypes

The presence of multiple endogenous ligands led to the suggestion that each ligand acted at a particular receptor subtype. Martin et al., 1976, reported the identification of μ- (from morphine) and κ- (from ketocyclazocine) opioid receptors in cross tolerance studies in dogs. Shortly after, Lord et al., in 1977, located a binding site with high affinity for enkephalins within the isolated mouse vas deferens, the delta (deferens {δ}) receptor.

Therefore pharmacological studies have defined at least three classes of opioid receptors termed δ, κ and μ that differ in their affinity for various opioid ligands and in their distribution in the CNS [Herz 1993]. The δ and μ receptors bind enkephalins and endorphins, the κ receptors potently bind dynorphins and the μ receptors selectively bind endomorphin-1 and endomorphin-2.

1.8.3.1. δ-Opioid Receptor Subtype

Two groups reported the isolation of cDNA clones encoding the mouse δ-opioid receptor [Evans et al., 1992; Kieffer et al., 1992]. Both groups screened a cDNA library from NG108-15 cells for the δ-opioid receptors. Monkey kidney (COS) cells transfected with δ receptors were screened for receptors that bound a radioactive δ-selective agonist. Between the groups, there was a difference in one amino acid. The mouse δ opioid clone isolated by Yasuda et al., 1993 was identical to mouse δ opioid receptor cloned by Evans and colleagues. Rat (97% identity sequence to the mouse) and human (93% amino acid identity

to both the mouse and the rat δ opioid receptors) δ opioid receptors have also been cloned by Fukuda et al., 1993 and Knapp et al., 1994 respectively.

The report by Yasuda et al., also illustrated that the cloned mouse δ receptor has a much higher affinity for Met- and Leu-enkephalin than the dynorphins. δ-selective agonists bound to the cloned δ receptor whereas κ and μ did not, and the cloned receptor had a high affinity for δ-selective antagonists compared to a lower affinity for κ-selective antagonists [Reisine and Bell 1993]. Evans et al., 1992, Kieffer et al., 1992 and Yasuda et al., 1993 all reported that the cloned δ opioid receptor was coupled to pertussis toxin-sensitive G-proteins to mediate agonist inhibition of cAMP formation, indicating that it is functionally coupled to adenylyl cyclase. Following the discovery that μ receptor ligands inhibit the binding of δ ligands in a competitive and non-competitive manner, Rothman and colleagues, 1988 divided δ-receptors into two subtypes; association with μ ($\delta_{complexed}/\delta_{cx}$) and non-association with μ ($\delta_{noncomplexed}/\delta_{ncx}$). Pharmacologically Traynor and Elliott 1993, have implied that the δ opioid receptor may exist as δ_{ncx}/δ_1 and δ_{cx}/δ_2, however the peripheral δ receptor in the mouse vas deferens does not conform to this classification [Wild et al, 1991]. There is at this time no structural evidence from cloning studies for the existence of subtypes (Table 1.5).

1.8.3.2. κ-Opioid Receptor Subtype

Yasuda et al., 1993 isolated and functionally characterised a 380amino acid protein from mouse brain corresponding to the κ opioid receptor. It was reported to have a 61% amino acid identity with the sequence of the mouse δ receptor. Functional studies illustrated that the cloned mouse κ receptor expressed in COS cells had a high affinity for dynorphin and κ agonists (U69593 and U50488) and a low affinity for the enkephalins. Several groups have cloned a 380 amino acid rat κ opioid receptor [see Knapp et al., 1995]. A guinea pig κ opioid receptor has also been cloned with ~89% amino acid identity to the mouse and rat κ opioid receptors [Xie et al., 1994]. The cDNA for a human κ opioid receptor encoding a 380 amino acid sequence with 91% amino acid identity to the cloned rat κ opioid receptor has also been identified [Mansson et al., 1994].

Like the δ opioid receptor the cloned κ receptor associates with pertussis toxin-sensitive G-proteins and mediates the inhibition of cAMP formation, and also mediates agonist inhibition of Ca^{2+} channel activity [Tallent et al., 1993]. Pharmacologically κ may exist as κ_{1a}, κ_{1b}, κ_2 and κ_3 [Cheng et al., 1995; Woolemann 1996], but there is no structural evidence from cloning studies for their existence (Table 1.5).

1.8.3.3. μ-Opioid Receptor Subtype

The first μ opioid receptor to be cloned was from a rat brain cDNA library by Chen et al., 1993. It consisted of 398 amino acids and had a ~64 and ~60% amino acid identity to the mouse δ opioid receptor and κ opioid receptor respectively. When expressed in COS cells Chen and colleagues reported a high affinity for the μ-selective agonist DAMGO and antagonists such as naloxone and cyprodime. The δ and κ selective agonists DPDPE and U50488 respectively, bound with low affinity. A human cerebral cortex cDNA library was screened by Wang et al., 1994 to isolate a human μ receptor clone of 400 amino acids.

The cloned μ opioid receptor mediated agonist inhibition of forskolin-stimulated cAMP formation indicating that it is coupled to adenylyl cyclase. Pharmacologically, μ may exist as μ_1, μ_2 and μ_3 [Pasternak and Wood, 1986, Cruciani et al., 1994], but there is no structural evidence from cloning studies for their existence (Table 1.5).

1.8.3.4. σ and ε-Opioid Receptor Subtype

Originally the sigma (σ) receptor was thought to be an opioid receptor, however this no longer holds for two reasons. The first was that the receptor mediated effects were not reversed by high concentrations of opioid receptor antagonists. And the second was that opioid receptors are enantioselective for (-)-isomers of opioid receptor agonists and antagonists, whereas σ receptors are enantioselective for the corresponding (+)-isomers [Walker et al., 1990]. The studies of the δ-opiate-receptor gene revealed a group of human intronless genes encoding homologs of the opiate receptor gene family, the ε-opioid receptor subtype. They share 60-70% amino acid identity with each other but only 36-39% with the μ, δ and κ receptors and do not bind opioids [Uhl et al., 1994].

1.8.4. Mechanism of Action

Opioid receptors belong to the family of guanine nucleotide-binding protein (G-protein) coupled receptors with seven membrane-spanning segments, preferentially coupling to pertussis toxin-sensitive G-proteins (G_i/G_o) [Lambert 1995]. Opioids close voltage-sensitive calcium channels (VSCCs) and activate an outward K^+ current resulting in hyperpolarisation. The membrane effects act to reduce neuronal excitability (increasing K^+ conductance producing hyperpolarisation of the membrane) and transmitter release (inhibition of Ca^{2+} entry) [Childers 1993; Atcheson and Lambert 1994]. They act to inhibit the enzyme adenylate cyclase therefore reduce intracellular cAMP directly, and subsequently inhibit the voltage dependent K^+ channel, I_H [Ingram and Williams 1994] (Table 1.5).

1.8.5. Pharmacological Effects of Opioid Agonists

The central pharmacological effects of opioid agonists include; analgesia, respiratory depression, euphoria, nausea and vomiting and pupillary constriction. Supraspinal and spinal analgesia is produced by the action of morphine at the pharmacological μ_1 and μ_2 receptors respectively, whereas respiratory depression is produced via μ_2 receptors in the brain stem [Pasternak 1993]. μ-opioid receptor activation produces a subjective effect of euphoria, a high abuse potential and side effects that include respiratory depression. κ-opioid receptor activation produces a low abuse potential, subjective effects of dysphoria/psychotomimesis and above all does not appear to cause respiratory depression [Millan 1990]. The site of action responsible for nausea and vomiting is the area postrema (chemoreceptor trigger zone-CTZ), a region of the medulla where chemical stimuli may initiate vomiting. Vomiting is usually prevented by the administration of naloxone suggesting that it is an opioid mediated event. Pupillary constriction is caused by μ- and κ-receptor-mediated stimulation of the oculomotor nucleus. Peripheral pharmacological effects include; an increase in tone and a reduction in motility in many parts of the GI tract resulting in constipation (mediated by μ-receptors), histamine release from mast cells, hypotension and bradycardia [Rang, Dale and Ritter 1995] and diuresis (mediated by κ-receptors) [Atcheson and Lambert 1994] (see Table 1.5).

Table 1.5. Characteristics of μ, δ and κ opioid receptors.

	Opioid Receptors		
	μ	δ	κ
Endogenous Ligand	Endomorphin-1 (high affinity) β-Endorphin (low affinity) Enkephalin (low affinity)	Enkephalin	Dynorphin
Selective Agonist	DAMGO	DPDPE DSLET	ICI-199,441 U50,488H U69,593 Enadoline
Selective Antagonist	CTOP Cyprodime	Naltrindole	nor-BNI
Clinically Selective Agonist	Morphine Fentanyl	None	None
Adenylate Cyclase (I_H)	Inhibits	Inhibits	Inhibits
Voltage-dependent Ca^{2+} Channels	Inactivates	Inactivates	Inactivates
Inwardly Recifying K^+ Conductance	Increases	Increases	Increases
Pharmacological Receptor Subtypes	μ_1, μ_2, μ_3	δ_1, δ_2	κ_{1a}, κ_{1b}, κ_2, κ_3
Function	Analgesia Constipation Respiratory depression	Analgesia Respiratory depression?	Analgesia Diuresis Dysphoria Respiratory depression?
Cloned	Yes	Yes	Yes
Cloned Receptor Subtypes	None	None	None

Key: DAMGO = [D-Ala^2,MePhe^4,Gly(ol)^5] enkephalin, DPDPE = [D-Pen^2,D-Pen^5] enkephalin, DSLET = [D-Ser^2,Leu^5,Thr^6] enkephalin, nor-BNI = norbinaltorphimine, CTOP = D-Phe-Cys-Tyr-Orn-Thr-Pen-Thr-NH^2.

1.8.6. Effects of Opioids on Neurotransmitter Release

Presynaptically opioid agonists supress the release of many neurotransmitters including: noradrenaline, acetylcholine, GABA [Leslie 1987] and substance P from primary afferent fibres [Kuraishi et al., 1983]. Postsynaptically opioid agonists produce hyperpolarisation, induced by activation of K^+ conductances in neurons of the superficial dorsal horn directly [Miyake et al., 1989], and via an inhibition of the enzyme adenylate cyclase [Childers 1993; Atcheson and Lambert 1994] reducing intracellular cAMP and inhibiting the voltage dependent K^+ current I_H [Ingram and Williams 1994].

As glutamate is present in the terminals of small diameter afferent fibres and in dorsal horn interneurons [Battaglia and Rustioni 1988; De Biasi and Rustioni 1988], it is not surprising that glutamate has been shown to play an important role in pain transmission in the spinal dorsal horn [Okano et al., 1993]. Ueda et al., 1993 demonstrated that capsaicin induced the release of neuropeptides such as substance P, and evoked the release of glutamate in an extracellular Ca^{2+}-dependent and tetrodotoxin-insensitive manner, mainly from primary afferents in spinal dorsal horn slices. In addition there are reports of opioid inhibition of glutamate release although this is extremely controversial (see Chapter 5).

1.9. Nociceptin

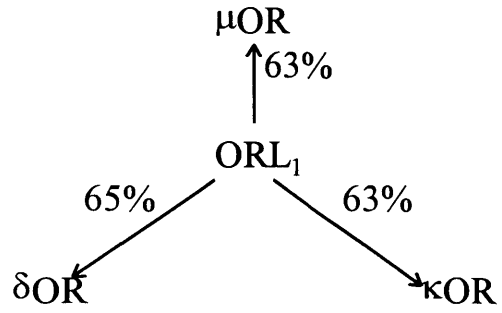
1.9.1. Orphan Receptor

Cloning studies in 1994 and 1995 revealed an atypical opioid receptor not belonging to the classical opioid receptors (μ, δ or κ) [Bunzow et al.,1994; Mollereau et al.,1994; Fukuda et al., 1994; Chen et al., 1994; Wang et al., 1994; Wick et al., 1994; Lachowicz et al., 1995], termed the orphan receptor. This orphan receptor did not show high affinities for μ, δ or κ opioid ligands (see Table 1.6).

Isolated from human genomic cDNA it was termed $hORL_1$ by Mollereau et al., 1994 who stated that its closest relatives structurally and functionally were the G-protein coupled opioid receptors, sharing ≤65% homology to the μ-,δ- and κ-opioid receptors in the transmembrane region (see Fig 1.10). They concluded that the $hORL_1$ receptor was a new member of the opioid receptor family with a potential role in modulating a number of brain functions, including instinctive behaviour and emotions. They also reported that the

orphan receptor expressed in CHO cells inhibited forskolin-induced accumulation of cAMP when activated by the opiate etorphine. Etorphine was ~3 orders of magnitude less potent to inhibit adenylate cyclase by the $hORL_1$ than at the opioid receptor. This effect could not be produced by the endogenous opioid peptides nor by selective μ-,δ- and κ-opioid agonists acting at the $hORL_1$ receptor [Mollereau et al., 1994; Wang et al., 1994; Lachowicz et al., 1994] (Table 1.6).

Fig 1.10. The transmembrane domain homology between the ORL_1 receptor and μ-, δ- and κ-receptors.



Bunzow and colleagues reported the isolation of a 3.1kb cDNA from a rat brain library, they called LC132. Comparison of the amino acid sequences of LC132 with μ-,δ- and κ-opioid receptors revealed that 145 amino acids were conserved among all four receptors, resulting in a 45% homology, however when the transmembrane domains were compared for homology, LC132 was ~64% identical to the rat μ, rat κ and mouse δ opioid receptors. As the four receptors possess extensive conservation in their first, second and third putative cytoplasmic loops, they deduced that LC132 probably couples to the same second messenger pathways, i.e. inhibition of adenylyl cyclase, activation of inwardly rectifying K^+ channels and inhibition of N-type calcium channels. The dense expression of LC132 mRNA (sharing a 92% homology to $hORL_1$) in limbic, cortical and spinal cord nuclei was consistent with the abundance of receptors that mediate analgesia and the perception of pain (Table 1.6). Other groups have cloned similar receptors (see Table 1.6).

Table 1.6. Summary of the cloned orphan receptors to date.

Reference	Receptor	Source	N° Amino Acids
Bunzow et al., 1994	LC132	Rat Brain	367
Mollereau et al., 1994	$hORL_1$	Human Brain	370
Fukuda et al., 1994	ROR-C	Rat Cerebrum	367
Chen et al.., 1994	-	Rat Brain	367
Wang et al., 1994	XOR1	Rat Brain	367
Wick et al., 1994	Hyp 8-1	Rat Brain	391
Lachowicz et al., 1995	C3	Rat Cerebral Cortex	367

1.9.2. Endogenous Orphan Receptor Ligand

In 1995 two separate reports by Meunier et al. and Reinsheid et al. described the identification of the endogenous ligand for ORL_1. Meunier and colleagues isolated a neuropeptide from rat brain, a heptadecapeptide, the sequence of which most closely resembled the opioid peptide, dynorphin (Fig 1.11). An synthetic peptide identical to the one discovered exhibited nanomolar affinity in the recombinant ORL_1 mediated adenylate cyclase inhibition. The natural peptide shared structural similarity with dynorphin and the sequence was present as a large precursor whose mRNA was expressed in the brain. When administered intracerebroventricularly to mice it induced hyperalgesia, therefore as its actions appear to increase reactivity to pain, Meunier and colleagues named the new peptide nociceptin.

Reinscheid et al., 1995 identified from homogenates of porcine hypothalamus an endogenous ligand for the orphan G-protein coupled receptor LC132. It was termed Orphanin FQ, orphanin to denote its relation to the orphan receptor and FQ to specify it by the termini of its amino acid sequence, phenylalanine (F) and glutamine (Q) respectively. Orphanin FQ was found to potently inhibit forskolin stimulated cAMP accumulation with an IC_{50} of 1.05nM, and the application of opioid agonists or antagonists to the orphan

receptor did not affect this inhibition. Thus demonstrating that orphanin FQ appeared to be pharmacologically and physiologically distinct from opioid peptides.

Fig 1.11. The amino acid sequence of the heptadecapeptide, nociceptin or orphanin FQ and dynorphin. The shared residues are shown in bold typeface.

Orphanin FQ H-Phe-**Gly-Gly-Phe**-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-**Lys**-Leu-Ala-**Asn-Gln**-OH.
Dynorphin H-Tyr-**Gly-Gly-Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-**Lys**-Trp-Asp-**Asn-Gln**-OH.

1.9.3. Pharmacological Effects of Nociceptin

The original finding of Meunier and colleagues was that nociceptin induced hyperalgesia in mice when administered intracerebroventricularly, therefore its actions appeared to increase reactivity to pain. There are now reports to suggest that orphanin FQ is a functional anti-opioid peptide [Mogil et al., 1996A & B]. Orphanin FQ was injected intracerebroventricularly into mice by Mogil and colleagues, 1996A, where it did not produce hyperalgesia as previously stated but reversed opioid mediated stress induced antinociception. In the same report orphanin FQ dose-dependently reversed the systemic antinociception produced by morphine. In addition, Mogil et al., 1996B demonstrated that orphanin FQ could completely block supraspinal antinociception produced by DAMGO (μ specific agonist), DPDPE (δ specific agonist) and U-50,448H (κ specific agonist) and had no effect on nociceptive sensitivity.

Stanfa et al., 1996 demonstrated that nociceptin selectively modulates spinal nociceptive events by dose-dependently reducing C-fibre wind-up and post discharge of the spinal dorsal horn neurons of the rat. The effects of nociceptin were reversed by 50μg but not 10μg of naloxone.

As the orphan receptor ORL_1 was abundant in limbic and hypothalamic structures [Mollereau et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995], Devine et al., 1996 suggested that the orphan system might be implicated in emotional and motivational functions. They concluded that as orphanin FQ, even at doses that disrupted motor behaviour, failed to produce motivational effects, this novel peptide did not possess abuse liability unlike the potential for abuse that is inherent with classical opiates [Wise 1989].

Also, as orphanin FQ failed to produce place preference or aversion in rats, the orphan system would appear not to participate in motivational actions.

1.9.4. Effects of Nociceptin on Ion Channels

As the dorsal raphe nucleus expresses high levels of ORL_1 but has not been reported to express μ, δ or κ opioid receptors, Vaughan and Christie, 1996 studied the action of nociceptin on K^+ channels at these neurons. Nociceptin was found to potently increase the inwardly rectifying K^+ conductance, with an EC_{50} of ~12nM. The response was naloxone insensitive. Connor et al., 1996A also reported coupling of nociceptin to a potassium conductance in rat locus coeruleus neurons with an EC_{50} of 90nM. This increase was weakly antagonised by naloxone, but not by CTAP (μ opioid antagonist), nalorphine (opioid antagonist) nor CPP (somatostatin antagonist).

When examining the effect of nociceptin on Ca^{2+} channel currents, Connor et al., 1996B reported that nociceptin produced a concentration dependent inhibition of the N-type Ca^{2+} channel currents (IC_{50} of 42nM) in the human neuroblastoma cell line SH-SY5Y. This effect could not be reversed by either CTAP (μ selective opioid antagonist), naltrindole (δ selective opioid antagonist) nor naloxone (non-selective opioid antagonist), but were blocked by pertussis toxin pre-treatment.

1.10. General Anaesthesia (see also Chapter 6)

Anaesthetic agents are given systemically (either intravenous or via lungs) and exert their main effects on the CNS. For an agent to be used as a general anaesthetic it must be readily controllable, so that induction and recovery are rapid and the level of anaesthesia can be adjusted as required during the course of the operation. There is evidence that several anaesthetics preferentially depress the ascending reticular activating system (RAS) in the brainstem. Activity in the RAS is concerned with maintaining consciousness, but this is not the only area of the brain depressed [Bowman and Rand 1990].

1.10.1. Inhalation and Intravenous Anaesthetic Agents

General anaesthetics are divided into intravenous and inhalation agents. Inhalation agents are primarily used for the maintenance of anaesthesia whereas the intravenous agents are

mainly used for induction. Inhalation agents do not act as rapidly as the intravenous agents. Among the inhalation agents are: halothane, isoflurane, enflurane, desflurane, sevoflurane and nitrous oxide. Intravenous agents act rapidly, producing unconsciousness within about 20secs. In general as intravenous anaesthetic agents are eliminated from the body at a lower rate than the inhalation agents and as they do not provide an adequate level of anaesthesia (with the exception of propofol) they are unsatisfactory for maintaining anaesthesia. They include, thiopentone, etomidate, ketamine and propofol.

1.10.2. Mechanism of General Anaesthesia

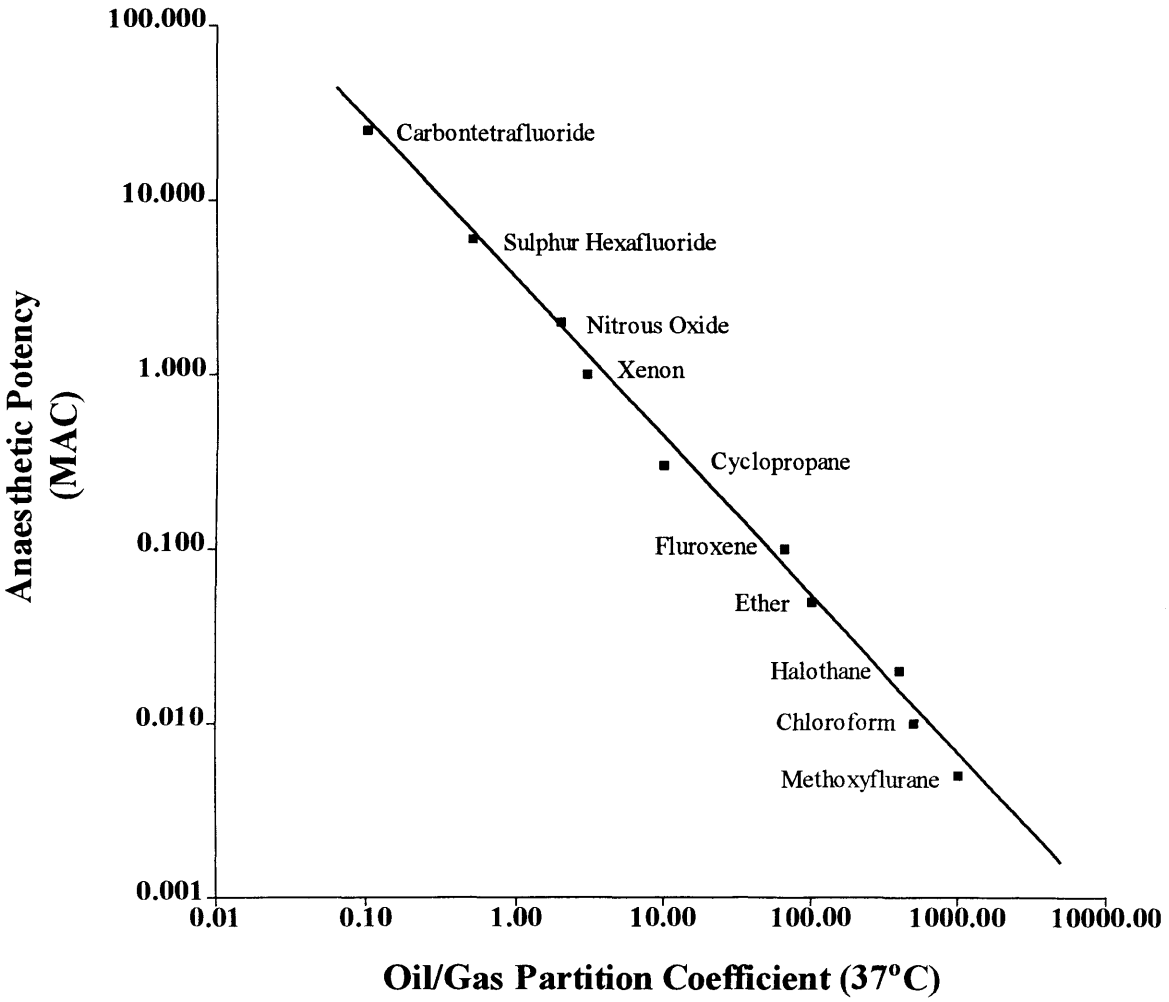
The molecular basis of the action of most general anaesthetic agents is at this stage unclear. However it is a commonly held view that general anaesthetic agents have a more pronounced effect on synaptic mechanisms in the CNS than on the propagation of electrical signals along axons [Larrabee and Posternak 1952; Griffiths and Norman 1993; Franks and Lieb 1994]. It is also apparent that anaesthetics appear to act principally on the cell membrane, where the theories of anaesthesia focus on the interactions with the two main components of the membrane, lipids and proteins [Franks and Lieb 1994].

1.10.2.1. Lipid Theory of Anaesthesia

The lipid theory was derived from the work of Overton and Meyer between 1899 and 1901. They reported a close correlation between anaesthetic potency (measured in terms of the concentration needed to produce reversible immobilisation of swimming tadpoles) and lipid solubility (measured as the olive-oil:water partition coefficient) in a group of unreactive organic compounds. The relationship of anaesthetic activity (MAC) and lipid solubility has been repeatedly confirmed [see Halsey 1991] (see Fig 1.12). MAC is defined as the concentration of anaesthetic agents required to block movement evoked by a noxious stimulus in 50% of patients (clinical IC_{50}) and is inversely proportional to the oil:gas partition coefficient of an anaesthetic agent. Support for the lipid theory of anaesthesia also comes from the measurement of membrane fluidity. In a recent paper, Norman et al., 1997 reported a strong correlation of MAC for volatile agents with change in membrane fluidity. However the relationship was that of an 'inverse' Meyer Overton. The authors concluded that their findings did not support the hypothesis that volatile anaesthetic agents acted by increasing membrane microviscosity of the bulk lipid bilayer

to produce anaesthesia. A similar but weaker correlation between lipid solubility and anaesthetic potency for the IV agents was reported by Frenkel et al., 1993.

Fig 1.11. Correlation of anaesthetic potency (minimum alveolar concentration:MAC) with oil/gas partition coefficient [Meyer and Overton 1901].



1.10.2.2. Protein Theory of Anaesthesia

General anaesthetics can bind to protein molecules and enzymes, but does this binding result in anaesthesia? Anaesthetics inhibit the activity of the lipid -free enzyme, luciferase, a pure soluble protein [Franks and Lieb 1984], with a close correlation of anaesthetic potency and potency in inhibiting luciferase for a wide range of anaesthetic compounds. General anaesthetics have been shown to produce stereoselective actions on ion channels [Franks and Lieb 1991]. This non-specificity is often taken to imply an underlying non-

selectivity in the action of the agents on targets, in particular the molecular targets involved in the induction of general anaesthesia [Franks and Lieb 1993]. As general anaesthetic agents produce stereospecific actions on ion channels this provides evidence that the action of anaesthetic agents may be at specific sites [Dildy-Mayfield et al., 1996].

Application of anaesthetics leading to an unconscious state, could be due to an interaction with excitatory transmission, ligand gated ion channels and/or VSCCs (see Fig 1.13), each component being located in the plasma membrane.

1.10.2.2.1. Excitatory Transmission

Various studies have indicated that volatile anaesthetics inhibit glutamate receptor-activated responses at or below 1MAC [Berg-Johnsen and Langmoen 1992; Kendig et al., 1994], depress excitatory synapses [Puil and El-Beheiry 1990] and both intravenous and inhalation anaesthetic agents have been reported to affect glutamatergic transmission inhibiting its excitatory actions [Bickler et al., 1995]. Although some anaesthetic agents may affect multiple sites, others influence specific receptors. For example ketamine and phencyclidine inhibit NMDA but not AMPA/kainate operated channels [Irifune et al., 1992].

1.10.2.2.2. Ligand Gated Ion Channels

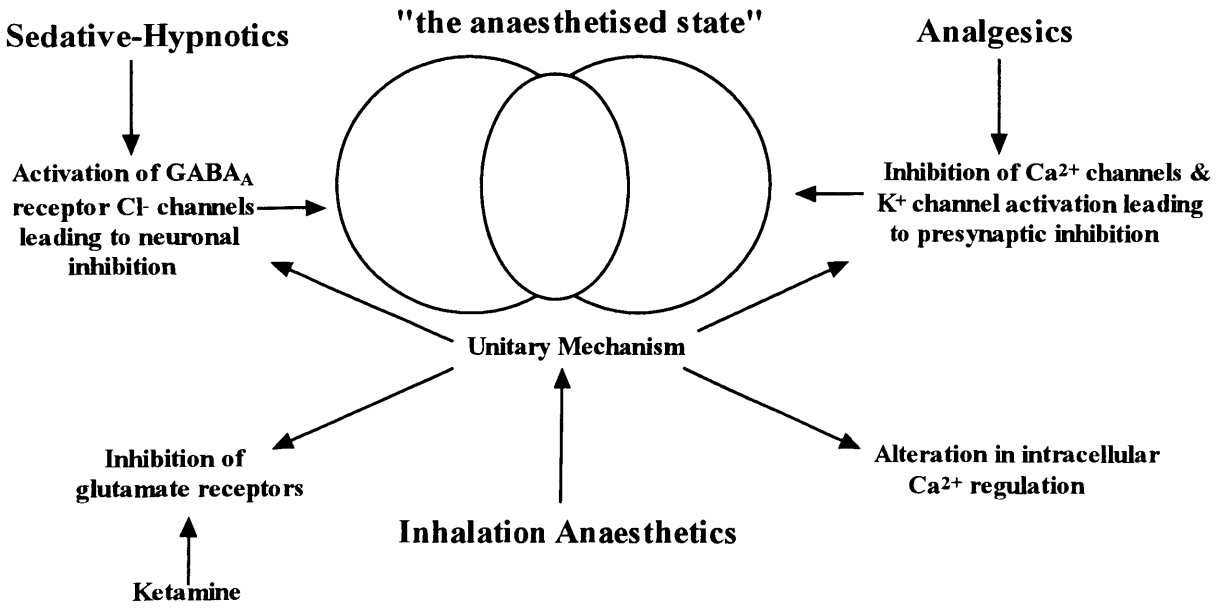
There is a good deal of evidence to support an interaction of a range of anaesthetic agents with the $GABA_A$ receptor potentiating its inhibitory actions [Tanelian et al., 1993]. In 1980, Collins reported the enhancement of GABA release following pentobarbitone. The binding of ligands to the $GABA_A$ receptor is modulated by barbiturates, increasing the affinity of GABA for the receptor dependent on the presence of Cl^-[Ashton 1983]. Following analysis, barbiturates appeared to increase the number of available GABA receptors and decreased the dissociation of GABA from the receptor [Asano and Ogasawara 1991; Willow and Johnston 1991]. At varience with the GABA hypothesis, Waller and Richter in 1980, reported an inhibition of GABA release by pentobarbitone. Volatile anaesthetics also interact with nicotinic and glycine receptors although the relevance remains to be determined [Bosnjak et al., 1988; Mascia et al., 1996].

1.10.2.2.3. VSCCs

Voltage sensitive Ca^{2+} channels have also been implicated as a target site for general anaesthesia [Lynch and Pancrazio 1994]. Study et al., 1994 demonstrated that isoflurane inhibited Ca^{2+} currents (generated by T, L, N and an additional unidentified type, probably P) at clinically relevant concentrations in hippocampal cells. Intravenous anaesthetic agents including, thiopentone, pentobarbitone, propofol, etomidate, alphaxalone and ketamine but not barbituric acid were reported to interact with the dihydropyridine binding site on neuronal L-type Ca^{2+} channels [Hirota and Lambert 1996A & B].

In summary, if a unitary target site exists, all the effects seen with individual anaesthetic agents should be identical. Yet different pharmacological effects are observed depending on the anaesthetic used, implying an interaction at multiple sites.

Fig 1.13. The major pathways for sedative-hypnotics and analgesics in generating sedation and analgesia, respectively, that can combine to produce the anaesthetic state [Lynch and Pancrazio 1994].



Aims

Glutamate is the predominant excitatory transmitter in connections to and from the cortex, and between cortical areas [Kaczmarek et al., 1997]. Glutamatergic transmission may be involved in the mechanism of general anaesthesia and is used by nociceptive afferent neurons. The excessive release of excitatory amino acids including glutamate has been implicated in the cause of excitotoxic damage to neurons and glia leading to neuronal death [Inglis et al., 1990; Bullock et al., 1991A & B; Kawamata et al., 1991]. Many human neurodegenerative diseases (epilepsy, brain ischaemia and amyotrophic lateral sclerosis) and head injuries have had glutamate mediated neuronal death associated with them [Rothstein et al., 1993; Davalos et al., 1997]. Drugs that antagonise the mechanisms of abnormal glutamate accumulation have been shown to reduce the infarct volume in experimental stroke [Hossmann 1994].

The primary aim of this thesis is to make a detailed and systematic examination of the role of analgesic/anaesthetic agents on glutamatergic transmission in the brain. There are two hypotheses under test:

i. Anaesthetic/analgesic agents decrease glutamate release to produce 'anaesthesia/analgesia'.

ii. Agents that reduce synaptic concentrations of glutamate may have neuroprotective properties.

Net synaptic concentrations of glutamate are a balance of uptake and release and the effects of anaesthetic agents and morphine on the uptake of glutamate in rat cerebrocortical and cerebellar synaptosomes will be studied. If anaesthetic agents and morphine altered the uptake of glutamate, any subsequent investigations on the release process would have to take into consideration the effect of these agents on the uptake of this neurotransmitter.

A reliable method for measuring glutamate release from a rat brain preparation would need to be developed to examine the effects of range of agents on the release of glutamate under basal and depolarising conditions.

Examination of the effects of μ-, δ- and κ-opioids and intravenous and inhalation anaesthetic agents on glutamate release from rat brain neuronal preparations will be studied in detail. Any effect on the depolarised release of glutamate would give clues to their mechanism of action and illustrate a role for these agents in glutamate induced neurotoxicity.

As glutamate release is enhanced by presynaptic NMDA receptor activation. An examination of the effects of a range of anaesthetic/analgesic agents on the binding of [^{3}H]MK-801 to NMDA receptors will be performed.

Collectively these studies presented in this thesis will address the hypothesis set out above.

Chapter 2. General Materials and Methods

Specific methodologies can be found in the appropriate chapters.

2.1. Sources of Chemicals

Sigma chemicals, Poole, Dorset, England.

Fura2-AM, Triton-X100, EDTA, EGTA, HEPES, DPDPE, DAMGO, naloxone, DMSO, morphine sulphate pentahydrate, glycine, glutamate, MK-801 hydrogen maleate, phosphoramidon, amastatin, bestatin, captopril, NADP, ketamine (racemic), barbituric acid, pentobarbital, choline chloride, lithium chloride, retinoic acid, n-heptane.

Fisons chemicals, Loughbourough, Leicestershire, England.

Sodium chloride, potassium chloride, magnesium chloride, magnesium sulphate, sodium hydrogen carbonate, sodium hydroxide, hydrochloric acid, methanol, sucrose, glucose, potassium dihydrogen phosphate, Folin and Ciocalteau's phenol reagent.

Gibco Life Technologies, Paisley, Scotland.

α-Minimum Essential Medium (α-MEM), fungizone, penicillin/streptomycin, foetal calf serum, new born calf serum, trypsin.

Semat Technical UK, Hertfordshire, England.

Spiradoline mesylate, propofol, cyprodime hydrobromide, L-trans pyrrolidine, 2,4-dicarboxylate (L-trans-PDC), nor-Binaltorphimine dihydrochloride (nor-BNI)

Radiochemicals.

[^{3}H]L-glutamic acid (specific activity 54Ci/mmol) greater than 98% pure was obtained from Amersham International PLC, Buckinghamshire, England.

[^{3}H]MK-801 (specific activity 23.9Ci/mmol) greater than 98% pure was obtained from NEN du pont.

Other chemicals and reagents

Nociceptin and ketamine(R- and S+) were kind gifts from Parke-Davis, Ann Arbor, MI, USA. Sodium Thiopentone (batch 62800) was a kind gift from Rhone-Poulenc Chemicals, Bristol, England.

Optiphase Hi-Safe 3 and Optiphase Safe-Life Technologies Ltd., Paisley, Scotland.

ICI-199,441- Tocris Cookson, Bristol, England.

Glutamate dehydrogenase- Novabiochem, Nottingham, England.

Percoll- Pharmacia Biotec Ltd., Hertfordshire, England.

Halothane- Zeneca Ltd., Cheshire, England.

Isoflurane- Abbott Laboratories, Kent, England.

Etomidate- Janssen Cilag, Ltd., Buckinghamshire, England.

2.2. Buffer Compositions

1. Bicarbonate buffer, in mmol litre^{-1} (glutamate release experiments)

NaCl 115, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 8.8 and oxygenated with 95% O_2, 5% CO_2 to pH 7.4.

2. HEPES buffer, in mmol litre^{-1} (glutamate uptake experiments)

NaCl 143, KCl 4.7, $CaCl_2$ 2.0, $MgCl_2$ 1.2, glucose 8.8 and HEPES 20, pH 7.4 with 10M NaOH.

3. Sucrose buffer, in mmol litre^{-1} (preparation of synaptosomes)

Sucrose 0.32 and HEPES 0.022, pH 7.4 with 10M NaOH.

4. Percoll Gradients (preparation of synaptosomes)

As Percoll is made of colloidal silica coated with polyvinyl-pyrrolidine, it was filtered prior to use with a 0.45μM sterile acrodisc filter to remove any silica aggregates. The pH of the Percoll was adjusted to 7.4 using HCl or NaOH just before dilution with sucrose buffer into: A: 23% filtered percoll + 77% sucrose buffer, B: 15% filtered percoll + 85% sucrose buffer, C: 10% filtered percoll + 90% Sucrose Buffer and D: 3% filtered percoll + 97% sucrose buffer. 1.8ml each of the four percoll solutions were layered onto 6, 10ml sorvall tubes in the descending order of percoll concentrations: A 23%, B 15%, C 10% and D 3%. The percoll gradients were stored at 4°C until use.

2.3. Rat Cerebrocortical and Cerebellar Synaptosomal Preparation

Female Wistar rats (200-250g) were killed by cervical dislocation and decapitation. The brain was rapidly removed and the cerebellum was placed in 4mls ice-cold sucrose buffer. The cortex was detached from its internal structures and placed in ice-cold sucrose buffer (10mls). Both the cortex and the cerebellum were homogenised using 10 up-down strokes at 700rpm of a glass Teflon tight-fit Potter S homogeniser. The homogenate was spun at 1,000g for 10mins at 4°C in a Biofuge 28RS Heraeus Sepatech centrifuge and the supernatant removed. The supernatant was pipetted gently onto six prepared percoll gradients (A, B, C and D in Sucrose Buffer), and centrifuged at 20,000g for 10mins at 4°C [Dunkley et al., 1988]. After centrifugation, fractions 3 and 4 (corresponding to interfaces 15 and 23%) were pipetted into a cold plastic tube and the mixture washed twice (by centrifugation at 20,000g/4°C/10mins) with HEPES buffer. The final synaptosomal pellet was resuspended in 1ml of HEPES buffer.

2.4. Rat Cerebrocortical and Cerebellar Slice Preparation

The brain from female Wistar rats was dissected as described previously, in Section 2.3, and placed in 20mls ice-cold oxygenated bicarbonate buffer. The outer cortex was removed from its internal structures, and the cerebellum was placed to one side. Both structures were cut at 350μm, turned at right angles and cut again at 350μm, and suspended in bicarbonate buffer. The slices were washed three times in fresh bicarbonate buffer, then agitated in a shaking water bath set at 37°C for 40mins. The slices were handled as described in Chapter 4, Section 4.5, page 84.

2.5. Rat Cerebrocortical Membrane Preparation

Rat cerebrocortical slices were prepared as described previously in Section 2.4. 5mls HEPES-EDTA buffer was added to the slices in a 50ml centrifuge tube, and homogenised using an Ultra Turrax set on high speed for 5x10 second bursts. The homogenate volume was made up to 40ml before centrifugation at 20,000g for 10mins at 4°C in a Biofuge 28RS Heraeus Sepatech centrifuge. The supernatant was removed and rehomogenised in 5mls of buffer. The homogenisation and centrifugation was repeated twice and the final membrane pellet was resuspended in 2mls HEPES-EDTA buffer per brain. Membranes were used as described in Chapter 7, Section 7.2, page 138.

2.6. Tissue Culture of SH-SY5Y and P19 Cells

SH-SY5Y human neuroblastoma and undifferenciated P19 murine embryonal carcinoma cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% Foetal Calf Serum (FCS), 100iu/ml penicillin, 100iu/ml streptomycin, and 2.5μg/ml fungizone and α- MEM supplemented with 3.5% New Born Calf Serum (NBCS), 1.5% Foetal Calf Serum (FCS), 100iu/ml penicillin, 100iu/ml streptomycin, and 2.5μg/ml fungizone respectively. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C. SH-SY5Y and P19 cells in monolayer cultures in plastic tissue culture dishes were maintained in the exponential growth phase by sub-culturing every 4days for SH-SY5Y cells, or 2days for P19 cells. Sub-culturing invloved the addition of 2mls trypsin (0.5g/l) (to dislodge the cells) to one flask of cells for 5mins followed by the addition of 8mls of media. The mixture was split into 10 other flasks containing 30mls of the supplemented media. To differentiate P19 cells into neurons, 10μM retinoic acid was added to the media. The cells were incubated for 2days, after which the media was removed and replaced with 25mls of the fresh relevant media. These cells were not passaged further.

2.7. Harvesting and Washing Proceedures of SH-SY5Y and P19 Cells

Confluent SH-SY5Y and differentiated P19 cells (following 14days incubation in media containing retinoic acid) were harvested using 5mls 50mM HEPES buffered saline (0.9%) containing 3mM EGTA. The cells were detached and resuspended in 15mls Krebs-HEPES buffer in a 15ml centrifuge tube. The suspension was inverted in order to break up clumps of cells, and sedimented at 1500rpm for 2mins. The addition of 15mls Krebs-HEPES buffer and the centrifugation was repeated twice for each 75cm^2 flask of cells.

2.8. Preparation of SH-SY5Y and P19 Cell Membranes

If cell membranes were needed, the cells were harvested as above, but resuspended in 5mls HEPES-EDTA buffer, and homogenised using an Ultra Turrax set at high speed for 5x10 second bursts, as in the preparation of rat cerebrocortical membranes, Section 2.5, page 43. The homogenate volume was made up to 40ml, with HEPES-EDTA buffer before centrifugation at 13000rpm for 10mins at 4°C in a Biofuge 28RS Heraeus Sepatech centrifuge. The supernatant was removed and rehomogenised in 5mls of HEPES-EDTA

buffer. The homogenisation and centrifugation was repeated twice and the final membrane pellet was resuspended in 5mls HEPES-EDTA buffer prior to use.

2.9. Measurement of Intra-cellular Calcium- $[Ca^{2+}]_i$ in SH-SY5Y and P19 Cells

Confluent SH-SY5Y (passage numbers 32-37) and differentiated P-19 cells (passage numbers 4-9) were harvested and washed in Krebs-HEPES buffer, as in Section 2.7, page 44. 3mls of cell suspension were loaded with fura-2 by incubating them for 30mins at 37°C in Krebs-HEPES buffer containing 3μM fura-2AM. Fura-2 is a Ca^{2+} fluorophore, and because of its electronegativity would not cross cell membranes. Fura-2 AcetoxyMethylesther (AM) is a membrane-permeable Ca^{2+}-insensitive ester of the Ca^{2+} fluorophore fura-2. Fura-2AM becomes Ca^{2+}-sensitive as it crosses the cells membrane when the AM group is cleaved by esterase enzymes within the cell membrane. This process is known as de-esterification, and leaves Fura 2 as a free acid trapped inside the cell and able to bind Ca^{2+}. After 30mins loading the cells were centrifuged at 37°C for 2mins at 1500rpm in a Heraeus Labofuge 400R centrifuge, and resuspended in Krebs-HEPES buffer for 20mins at room temperature to enable complete de-esterification of the Fura 2AM. The cells were re-sedimented twice and finally resuspended in 6mls Krebs-HEPES buffer. 2mls of cell suspension was placed in a quartz cuvette containing a magnetic stirrer, and the cuvette was placed into a cuvette holder maintained at 37°C with a water jacket.

The cells loaded with fura-2 were subjected to dual excitation at 340 and 380nm, with emission set at a single wavelength of 510nm (Fig 2.1). Under Ca^{2+} free conditions fura-2 excitation specrum peaks at ~380nm. When Ca^{2+} is added the spectrum shifts to the left with a new exciation maximum of ~340nm. Thus when Ca^{2+} increases in a cell a simultaneous increase in 340nm fluorescence and a decrease in 380nm fluorescence is observed. At any given time the 340/380 ratio is proportional to $[Ca^{2+}]_i$ as shown in the Grynkiewicz equation [Grynkiewicz et al., 1985];

$$[Ca^{2+}]_i = Kd \times (F_{min}/F_{max}) \times (R\text{-}R_{min}/R_{max}\text{-}R)$$

To calculate the $[Ca^{2+}]_i$ R_{max} and R_{min} (maximum and minimum fluorescence respectively) were calculated. R_{max} was measured upon the addition of 0.1% Triton-X100 causing the intensity curve at 340nm to increase and the recording at 380nm to simultaneously decrease. R_{min} was measured by additing 3mM EGTA (Ca^{2+} chelating agent), upon which the two curves cross. F_{max} and F_{min} are the corresponding fluorescence intensities at 380nm under stimulatory (+ Triton-X) and Ca^{2+} free (+ EGTA) conditions (see Fig 2.1).

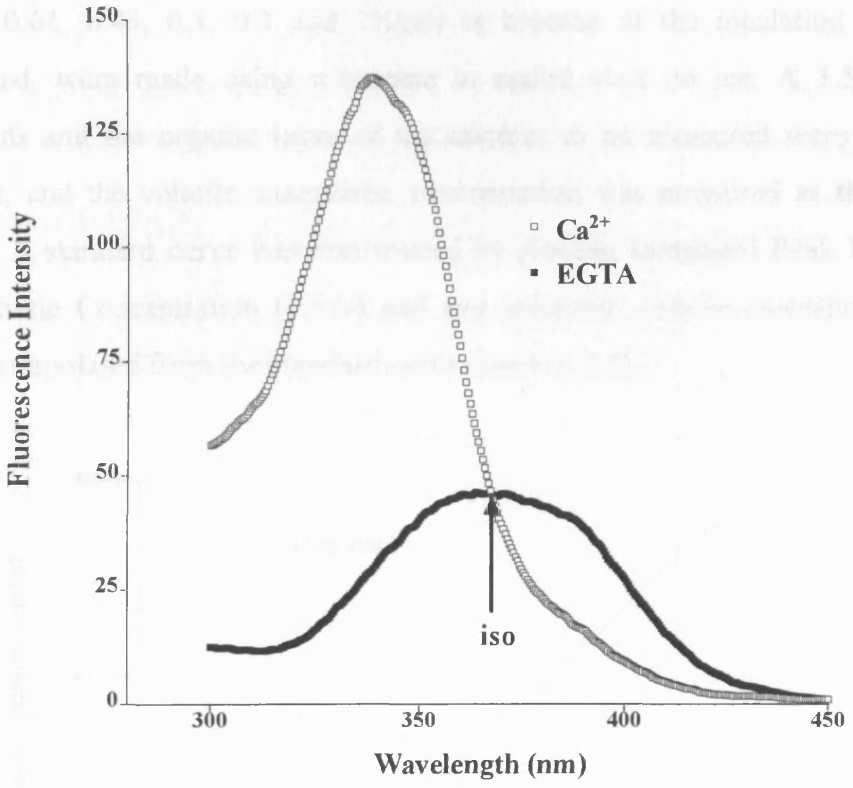


Fig 2.1. Fluorescence spectra of fura-2 free acid when bound and unbound to Ca^{2+}. The isobestic (iso) wavelength is where there is no net change in fluorescence between that in the presence of Ca^{2+} and EGTA.

2.10. Gas Chromatography (GC) [Rutledge et al., 1963]

Gas chromatography was used to determine volatile anaesthetic concentration delivered to the buffer and neuronal preparations. The Gas Chromatograph used was a Perkin Elmer 8410 with a 30m megabore DB-17 (Fisons) column and the results were monitored using a Perkin Elmer integrator model 8410, connected to the GC. Helium (20ml/min) was used as a carrier gas. Sample was detected using flame ionisation. The injector, detector and oven

temperatures were 140, 130 and 90°C respectively. The volatile anaesthetic was delivered to the buffer as described in Chapter 6, Section 6.2, page 113. At various times a 200µl sample of the buffer was taken using a gas-tight syringe, and added to an equal volume of n-heptane in sealed vials on ice (to prevent evaporation). n-heptane is used to extract the dissolved volatile from the aqueous buffer. n-heptane (the organic layer) and the buffer (the inorganic layer) are immiscible, the buffer settles at the bottom of the vial and n-heptane with the extracted volatile anaesthetic sits on top of the buffer. Standards (0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1%)v/v in heptane of the inhalation anaesthetic to be measured, were made using n-heptane in sealed vials on ice. A 1.5µl aliquot of the standards and the organic layer of the samples to be measured were injected onto the column, and the volatile anaesthetic concentration was measured as the integrated peak height. A standard curve was constructed by plotting Integrated Peak Height vs Volatile Anaesthetic Concentration (%v/v) and any unknown volatile anaesthetic concentrations were extrapolated from the standard curve (see Fig 2.2).

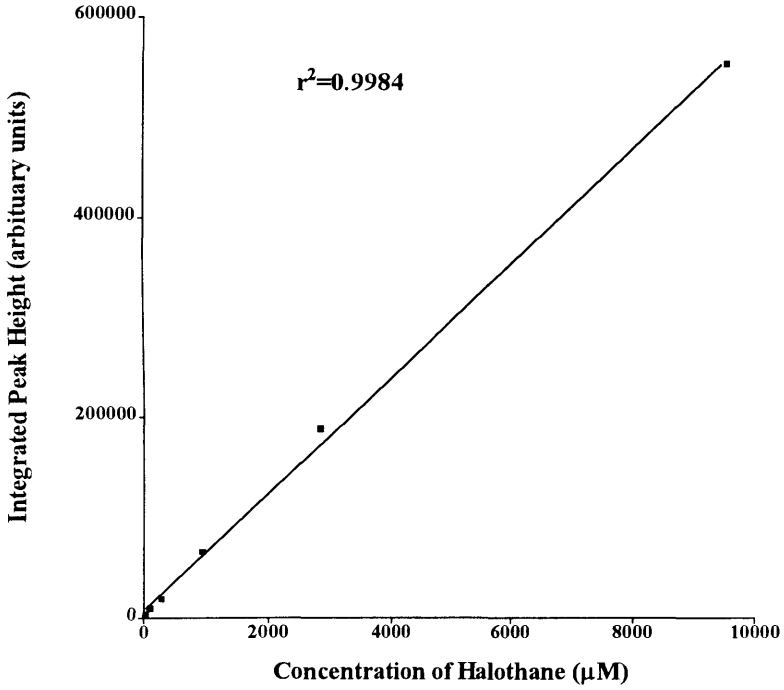


Fig 2.2. Typical standard curve for halothane determination. Integrated peak heights are plotted against the concentration of halothane. This data is from one experiment but is a representative for >20. Similar data were obtained for isoflurane.

2.11. Lowry Protein Assay [Lowry et al., 1951]

Any tissue to be assayed was diluted 1:10, 1:20 and 1:50, corresponding to 50, 25 and 10µl made up to 500µl in 0.1M NaOH to alkalinate the buffer and also to extrapolate the unknown protein concentrations from the standard curve. Bovine Serum Albumin (BSA) protein standards (0, 50, 100, 150, 200 and 250µg/ml) were also made up in 0.1M NaOH. The protein assay reagents were: Protein A: 2% Na_2CO_3 in 0.1M NaOH, Protein B: 1% $CuSO_4$ and Protein C: 2% Na^+K^+ tartate. A mixture of the three solutions was made, in the ratio of 100A : 1B : 1C. Of this mixture, 2.5mls was added to each tube containing 0.5ml volumes of the unknown protein samples and the standards, and incubated at room temperature for 10mins. A 1:4 dilution of Folins phenol reagent was made in H_2O from which 250µl was added to each tube, vortexed, and incubated at room temperature for 30mins to allow the blue colour to develop. 3ml disposable cuvettes were used to measure the absorbance readings at 750nm in a Corning Spectrophotometer. A standard curve was generated from the BSA protein standards using a linear regression fit from Graphpad Prizm 2, from which any unknown amounts of protein could be extrapolated (see Fig 2.3) [Lowry et al., 1951].

2.12. Data and Statistical Analysis

All data are presented as mean±Standard Error of the Mean (SEM) (n), where n=number of individual experiments, unless otherwise stated. Computer-assisted curve fittings were performed using Graphpad Prism 2. The analysis of dose response curves in uptake and release studies was performed by fitting a sigmoidal curve with a variable slope, setting the bottom of the curve at zero. Binding studies were analysed as described in Chapter 7, page 138 When appropriate statistical analysis was carried out using Wilcoxon rank sum test, with significance set at $*p<0.05$, following Kruskal Wallis where appropriate.

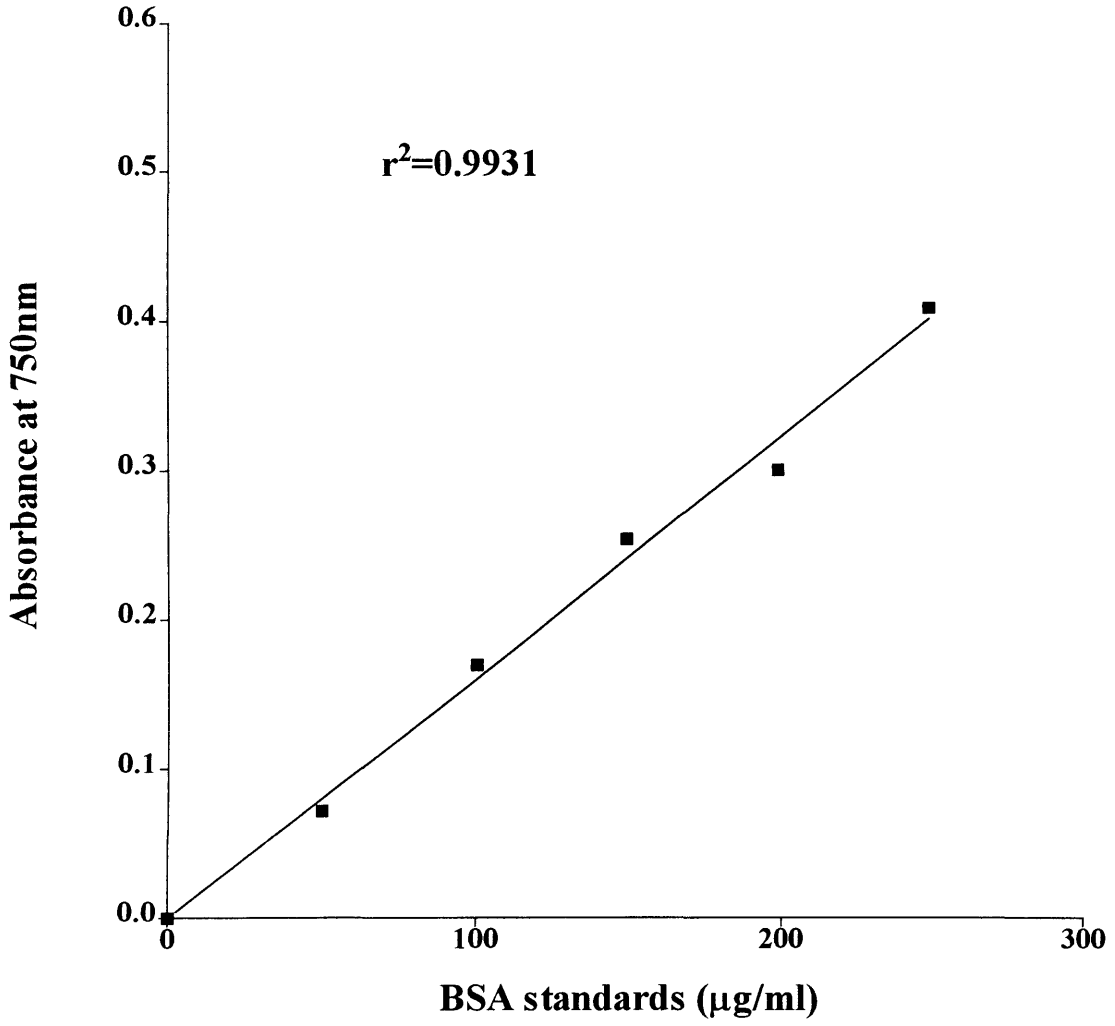


Fig 2.3. Representative protein assay standard curve, where absorbance at 750nm is directly related to the amount of the protein.

Chapter 3. Characterisation of the Glutamate Uptake Process into Cerebrocortical and Cerebellar Synaptosomes.

3.1. Introduction

Glutamate is the major excitatory neurotransmitter in the brain acting on NMDA, AMPA, kainate and metabotropic receptors [Kaczmarek et al., 1997]. Net synaptic glutamate concentrations result from a balance between release and re-uptake, and potential secretagogues may affect either or both of these processes. Uptake of glutamate plays a vital physiological role in terminating glutamatergic neurotransmission, and maintenance of constant and low levels of glutamate in the external environment, thereby limiting the neurotoxic effects of high synaptic glutamate concentrations [Erecinska 1987]. Excess glutamate release has the potential to produce excitotoxicity [Rothstein et al., 1993]. High synaptic glutamate concentrations achieved by impairment of glutamate transport have been implicated in epilepsy, brain ischaemia and amyotrophic lateral sclerosis [Rothstein et al., 1993]. These neurodegenerative diseases represent real clinical problems, that may be exacerbated by inappropriate use of anaesthesia and analgesia.

The properties of plasma membrane glutamate transport systems has evolved to the identification of four distinct glutamate transporters (see Chapter 1, Section 1.7, page 17). Four transporters are termed GLT-1, EAAC1, GLAST1 and EAAT and the characteristics are summarised in Table 3.1.

Table 3.1 Characteristics of high affinity glutamate transporters.

Glutamate Transporter	Size (N° amino acids)	Tissue Ditribution.	Cellular Localisation	K_m for L-glutamate (μM)
GLT1	573	Brain	glia	~2
EAAC1	524	Periphery Brain	neurone	12
GLAST	543	Brain	glia	77
EAAT	568	Brain	neurone	~3

Aims

The aims of this chapter are to characterise the uptake process of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes, and to examine the effects of a range of commonly used anaesthetic and analgesic agents on this process. The specific hypothesis being, that a decrease of glutamate uptake would lead to an enhancement of synaptic glutamate concentration. In future studies of glutamate release it will be important to confirm that the effects observed were not secondary to an interaction with the uptake transporters. Specifically a decrease in uptake would mimic release and an increase in uptake would mimic the inhibition of release.

3.2. Methods

Uptake of [^{3}H]L-glutamate into Synaptosomes

All studies were performed using either cerebrocortical or cerebellar synaptosomes prepared from rats as described in Chapter 2, Section 2.3, page 43. The uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes was performed after a 15min pre-incubation at 37°C of 100µl synaptosomes made up to 500µl with HEPES buffer. When examining the effects of various agents on [^{3}H]L-glutamate uptake, they were added to the synaptosomes and HEPES buffer for the duration of the pre-incubation. All reactions were initiated by the addition of 100nM [^{3}H]L-glutamate, and terminated by rapid vacuum filtration onto Whatman GF/B filters using a 12 place Millipore cell harvester. The radioactivity remaining on the filters was extracted using Optiphase "Safe" as a scintillant, and the activity quantified by liquid scintillation spectroscopy using a Packard 1900 TR Liquid Scintillation Analyser.

Characterisation of the Uptake Process

[^{3}H]L-glutamate was added to the synaptosomes for 10, 20 and 30secs, 1, 2, 5, 7, 10 and 15min. The contribution of diffusional uptake (i.e. non-specific) was determined by the incubation at 0°C. The synaptosomes and HEPES buffer were pre-incubated as stated previously prior to the addition of [^{3}H]L-glutamate for 1min. When investigating the Na^+ dependency of the glutamate uptake system, Na^+(143mM) present in the buffer was exchanged with either choline or lithium at 143mM. Determination of K_m (substrate concentration at which the uptake rate is half of its maximum), and V_{max} (maximum rate of the uptake), for the uptake of glutamate into cerebrocortical and cerebellar synaptosomes, was achieved by diluting 50nM [^{3}H]L-glutamate with increasing concentrations of unlabelled glutamate (150nM-500µM). The diluted/undiluted [^{3}H]L-glutamate was added to the synaptosomes and HEPES buffer for 1min.

As a positive control for the inihibition of glutamate uptake, the effect of a glutamate uptake inhibitor was examined. L-trans-pyrrolidine-2, 4-dicarboxylate (L-trans-PDC) (0.01-100µM) was pre-incubated with the synaptosomes prior to the addition of [^{3}H]L-glutamate. The reactions were conducted for 1min.

Effect of Anaesthetic Agents and Morphine

Synaptosomes were pre-incubated for 15min at 37°C as previously stated, in the presence of the intravenous anaesthetic agents:- propofol, thiopentone, ketamine (3, 10, 30, 100 and 300μM) and morphine (0.1-10μM). The inhalation anaesthetic agents isoflurane (3.6%, 2.70 rat MAC) and halothane (3%, 2.47 rat MAC) were delivered to the synaptosomes for 15min (pre-incubation) using a precalibrated Fluotec 3 vaporiser with humidified air (2.0 litre min^{-1} at 37°C) as the carrier gas. After the pre-incubation, [^{3}H]L-glutamate was added for 1 or 10min. [^{3}H]L-glutamate uptake was also performed under depolarising conditions (in the presence of 46mM K^+), with the highest concentration of the intravenous anaesthetic agents, the inhalation anaesthetic agents and morphine. When using propofol, the vehicle for propofol, Dimethyl Sulfoxide (DMSO) was used as a control.

Miscellaneous Methods and Data Analysis

Aqueous isoflurane and halothane concentrations were confirmed by gas chromatography, GC (Chapter 2, Section 2.10, page 46). Protein was measured by the method of Lowry et al., 1951 (Chapter 2, Section 2.11, page 48). Results are expressed as mean±sem (n). Statistical analysis was performed using Kruskal Wallis and Wilcoxon rank sum tests where appropriate, and considered significant when $p<0.05$.

3.3. Results

Time Dependence and Diffusional Uptake

As shown in Fig 3.1A & B, the uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes was time-dependent. In cerebrocortical synaptosomes Fig 3.1A, the uptake rose linearly from 27.14±4.69pmol/mg of protein at 10secs to 77.42±13.22 pmol/mg of protein at 1min. At 3min the uptake had reached a maximum of 94.2±14.55 pmol/mg of protein and declined to 73.42±9.28 pmol/mg of protein at 15min. In contrast, in cerebellar synaptosomes, Fig 3.1B, the uptake was less than that of the cerebrocortex. A gradual increase of [^{3}H]L-glutamate uptake was seen, increasing from 12.72±3.58 pmol/mg of protein at 10secs and reaching a steady plateau at 15min of 57.74±12.37 pmol/mg of protein. Diffusional uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes at 0°C was 8.28±1.99% and 16.78±2.03% of uptake measured at 37°C respectively.

Na^+-Dependence

The uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes was Na^+ dependent, as substitution of Na^+ (143mM) with lithium (143mM) or choline (143mM) significantly reduced uptake by 58.45±6.6 and 76.65±4.08%, and 62.39±0.4 and 81.52±3.13% in the cerebrocortex and cerebellum respectively (Fig 3.2).

Concentration Dependence

Cerebrocortical and cerebellar synaptosomal [^{3}H]glutamate uptake was concentration dependent. A Lineweaver-Burk plot of 1/v against 1/[s], (where v = velocity of the reaction and [s] = concentration of substrate) was constructed. Cerebrocortical synaptosomes had mean K_m (x-intercept = $-1/K_m$) and V_{max} (y intercept = $1/V_{max}$) values of 8.61±1.91μM and 1.71±0.30nmol/min/mg protein respectively, in the cerebellum the values were 2.23±0.33μM and 0.67±0.09nmol/min/mg protein respectively n=5 (Fig 3.3). The K_m and V_{max} values were significantly different by Wilcoxon Rank Sum test.

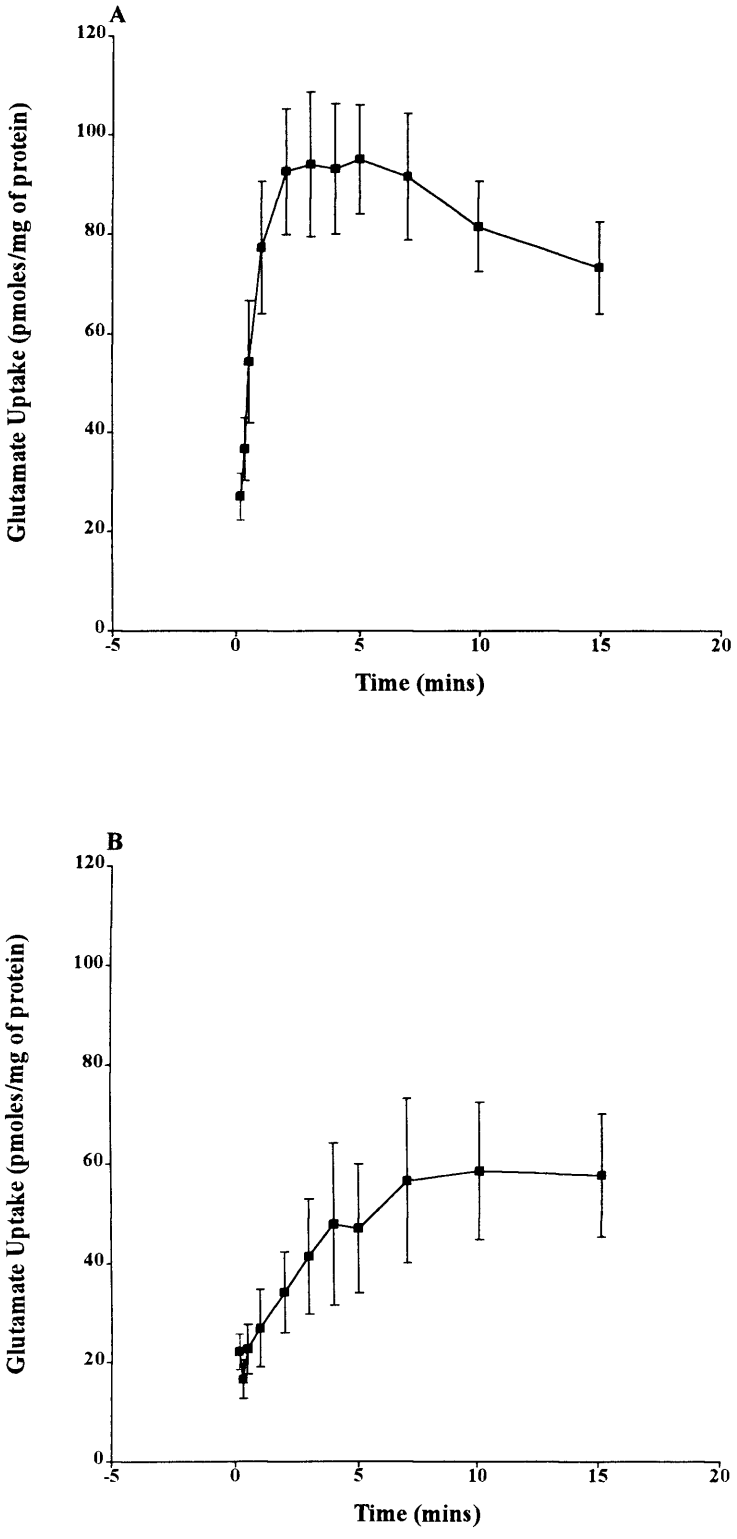


Fig 3.1. Uptake of [^{3}H]L-glutamate into cerebrocortical (A) and cerebellar (B) synaptosomes is time-dependent. Data are Mean±sem (n=5). The two time courses were significantly different by Kruskal Wallis * $p<0.05$.

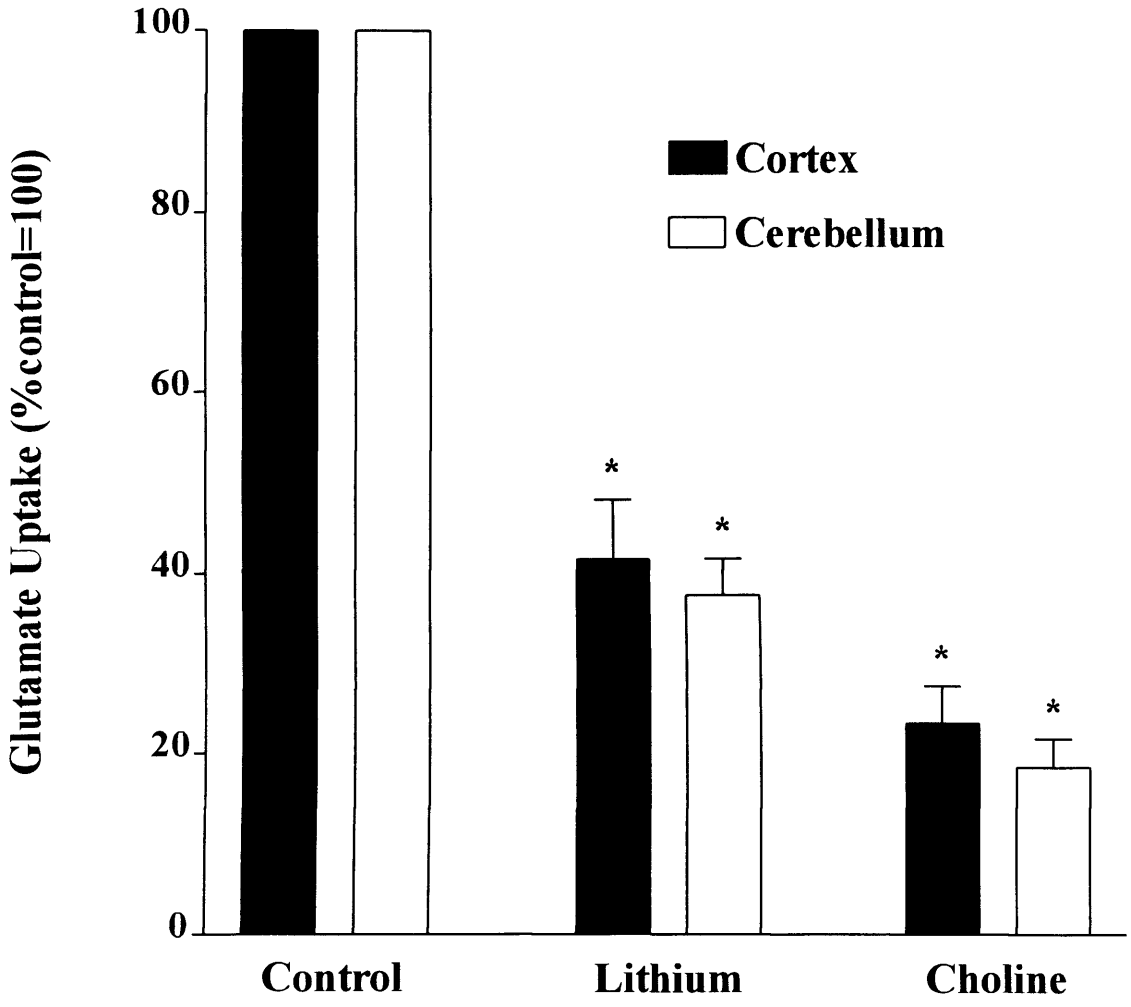


Fig 3.2. Substitution of Na^+ with lithium or choline within the HEPES Buffer reduced the uptake of [^{3}H]L-glutamate by 58.45±6.6 and 76.65±4.08%, and 62.39±4.04% and 81.52±3.13% in the cerebrocortex and cerebellum respectively. Data are mean±sem (n=5) and * illustrates statistical significance compared with control.

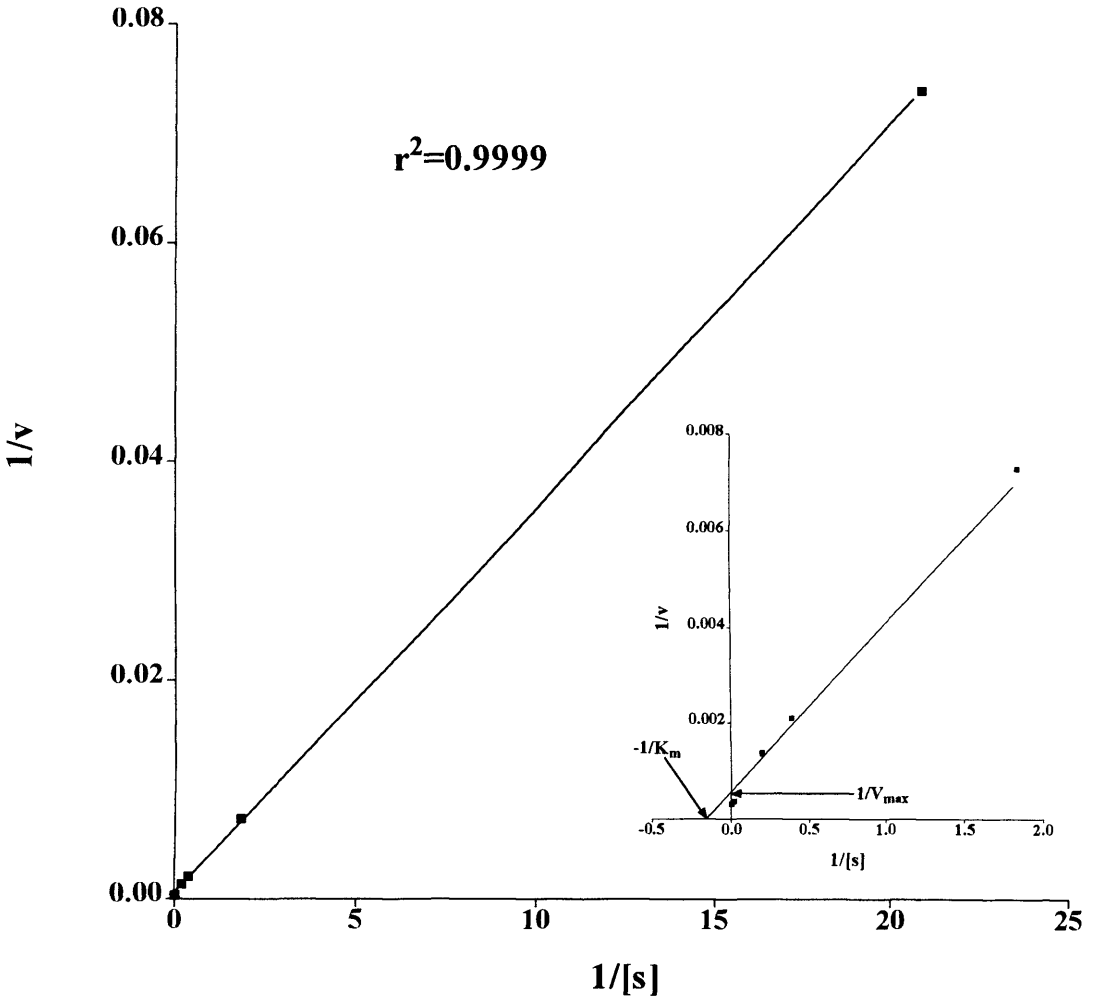


Fig 3.3. Lineweaver-Burk double reciprocal plots of 1/v (v=velocity of the reaction) against 1/[s] ([s]=concentration of substrate). The inset illustrates the x and y intercepts (expanded from the graph), from which the K_m and V_{max} values of 6.33μM and 1.798nM/min/mg of protein respectively were calculated. This data is from cerebrocortical synaptosomes and is representative of 5 experiments.

Effect of L-trans-pyrrolidine-2,4-dicarboxylate (L-trans-PDC)

L-trans-PDC inhibited the uptake of [^{3}H]L-glutamate into both cerebrocortical (Fig 3.4A) and cerebellar (Fig 3.4B) synaptosomes in a concentration dependent manner. A percentage inhibition of 88.84±1.78 and 82.4±7.05 was achieved at 100μM in cerebrocortical and cerebellar synaptosomes respectively, when compared to control. Further analysis of the dose response curve yielded IC_{50} values of 13.24 and 3.94μM for cerebrocortical and cerebellar synaptosomes respectively.

Effects of Anaesthetics and Morphine

Thiopentone and ketamine did not affect the uptake of [^{3}H]L-glutamate into cerebrocortical synaptosomes (Fig 3.5A). Propofol significantly inhibited the uptake in the cerebrocortex at 300, 100 and 30μM by 35.67±9.02, 22.33±7.65 and 13.6±5.6% respectively (Fig 3.5A). Thiopentone and propofol significantly reduced the uptake of [^{3}H]L-glutamate under depolarising conditions into cerebrocortical synaptosomes by 40±4.3 and 64.6±5.6% respectively. Ketamine proved ineffective (Fig 3.5B).

In cerebellar synaptosomes thiopentone and ketamine did not affect the uptake of [^{3}H]L-glutamate. Propofol significantly reduced the uptake by 63.4±5.7% and 53.6±13.49 at 300 and 100μM respectively (Fig 3.6A). Thiopentone and propofol significantly reduced the uptake of [^{3}H]L-glutamate under depolarising conditions into cerebellar synaptosomes by 29.6±7.0 and 70.8±2.4% respectively. Ketamine proved ineffective (Fig 3.6B).

Neither halothane (736±28μM), isoflurane (947±45μM) nor morphine (0.1, 1 and 10μM) affected the uptake of [^{3}H]L-glutamate into either cerebrocortical or cerebellar synaptosomes (Table 3.1).

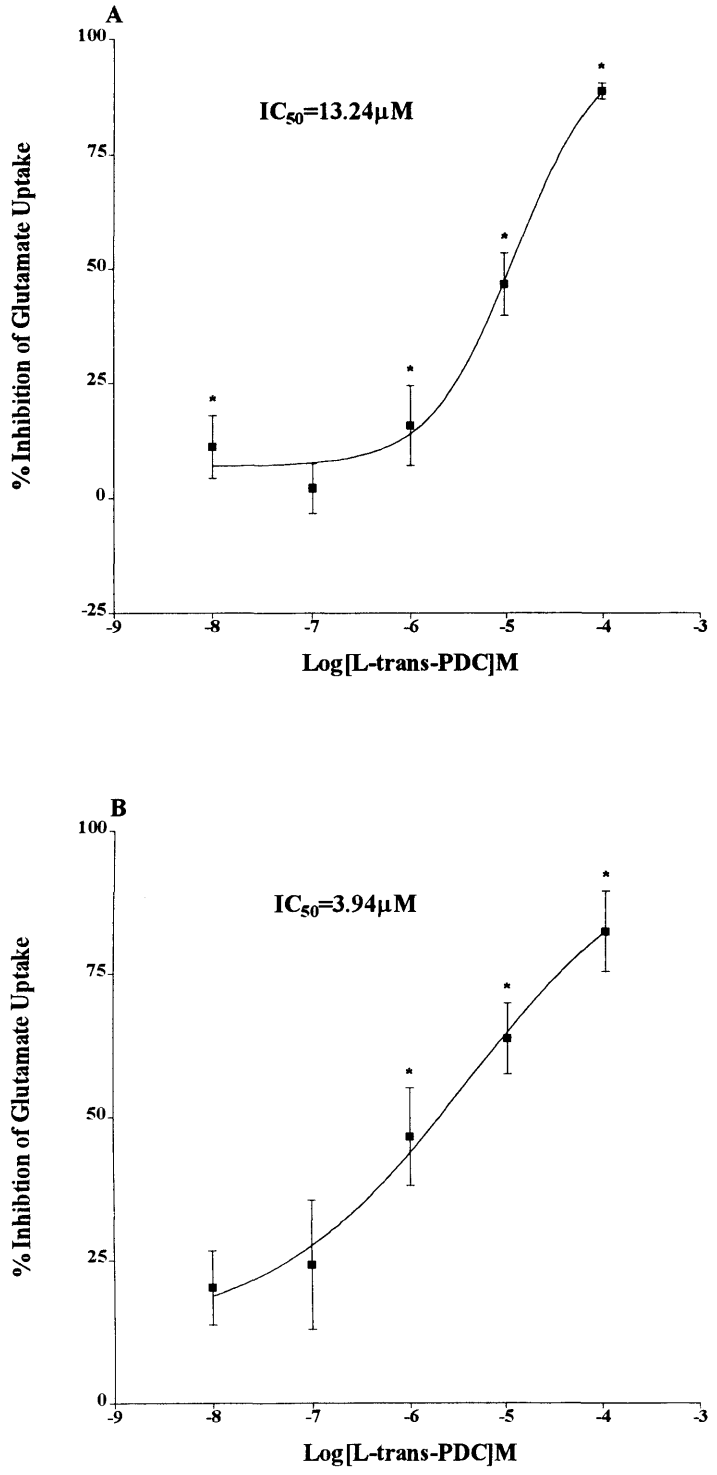


Fig 3.4. L-trans-PDC inhibited the uptake of [^{3}H]L-glutamate into cerebrocortical (A) and cerebellar (B) synaptosomes. Data are mean±sem (n=5-8). Dose-response curves were significantly different by Kruskal Wallis and * illustrates statistical significance compared with control.

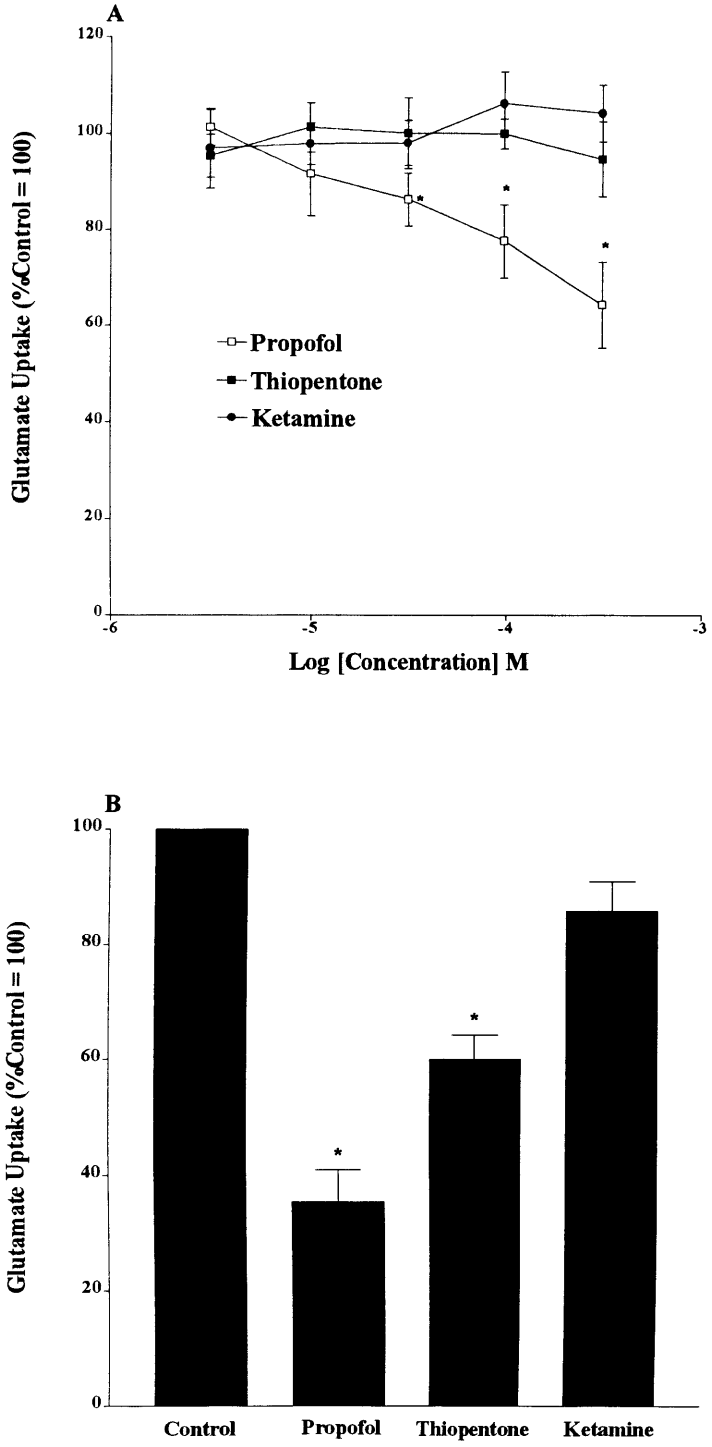


Fig 3.5. Propofol dose-dependently inhibited the uptake of [^{3}H]L-glutamate into cerebrocortical synaptosomes. Thiopentone and ketamine were ineffective (A). Propofol and thiopentone at 300μM inhibited the uptake of [^{3}H]L-glutamate under depolarising conditions into cerebrocortical synaptosomes. Ketamine proved ineffective (B). Data are mean±sem (n=5) and * illustrates statistical significance compared with control.

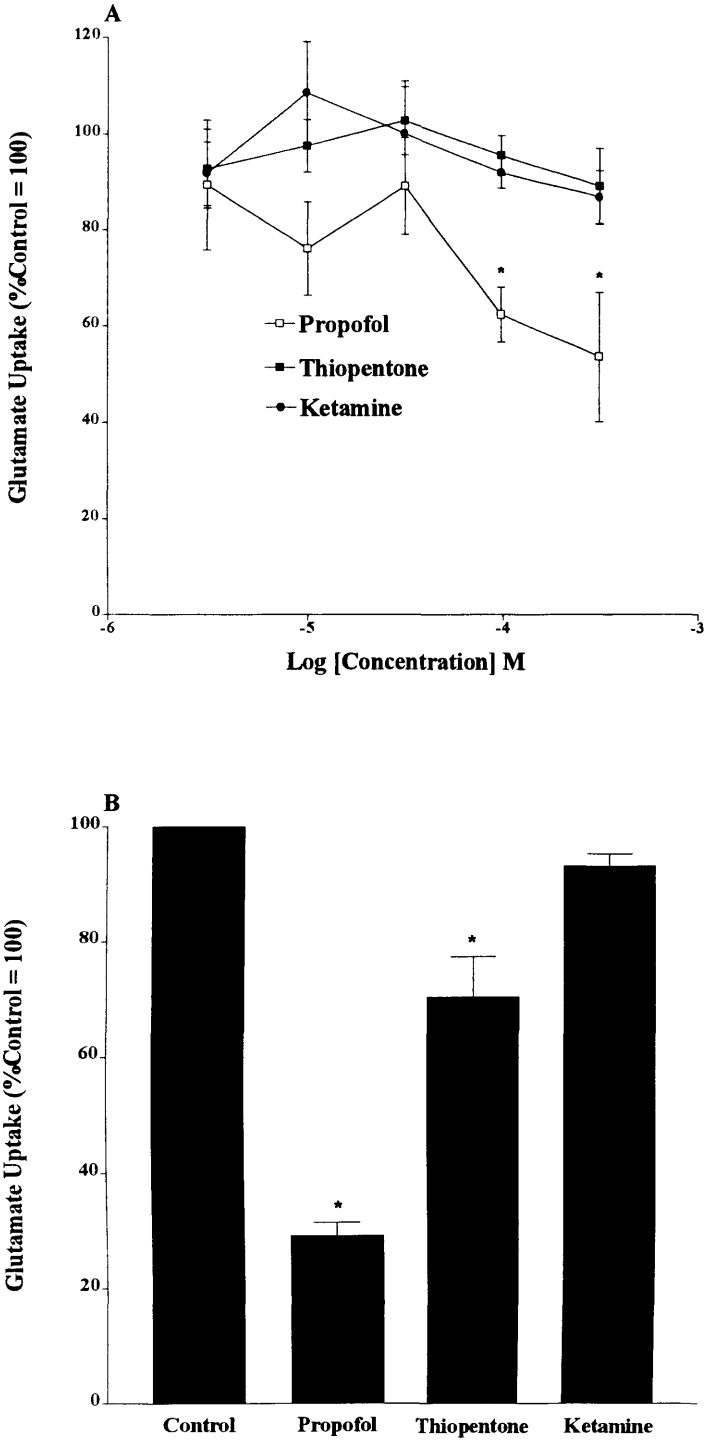


Fig 3.6. Propofol dose-dependently inhibited the uptake of [^{3}H]L-glutamate into cerebellar synaptosomes. Thiopentone and ketamine were ineffective (A). Propofol and thiopentone at 300μM inhibit the uptake of [^{3}H]L-glutamate under depolarising conditions into cerebellar synaptosomes. Ketamine proved ineffective (B). Data are mean±sem (n=5) and * illustrates statistical significance compared with control.

Table 3.1 Effects of isoflurane, halothane and morphine on [^{3}H]L-glutamate uptake into cerebrocortical (Ct) and cerebellar (Cb) synaptosomes. Data are mean±sem (n=5-6) for incubations terminated at 1min, unless otherwise stated. Results are expressed as % of control (100%). Controls for isoflurane and halothane are in the presence of the carrier gas, air.

	Glutamate Uptake (% control = 100)			
	Basal		**46mM K^+**	
	Ct	**Cb**	**Ct**	**Cb**
Isoflurane 947±45μM				
1min	92±7	81±9	103±7	111±16
10min	114±12	99±4	93±5	104±7
Halothane 736±28μM				
1min	104±11	106±8	103±7	100±10
10min	104±7	99±5	97±10	100±4
Morphine				
0.1μM	94±6	95±2	-	-
1μM	97±3	95±6	-	-
10μM	102±2	99±8	97±11	106±15

3.4. Conclusions

Rat cerebrocortical and cerebellar synaptosomes took up [^{3}H]L-glutamate in a time-, concentration, Na^+, temperature-dependent and L-trans-PDC sensitive manner. With the exception of propofol, neither inhalation and intravenous anaesthetic agents nor morphine altered the uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes. In cerebrocortical synaptosomes a linear relationship was observed up to 2min, after which the uptake reached a plateau. In cerebellar synaptosomes there was a more gradual rise in uptake reaching maximum at 10min. The results confirming the rapid uptake kinetics associated with the need to rapidly clear the synaptic cleft of released glutamate.

Substitution of Na^+ with either choline or lithium significantly reduced the uptake, consistent with the Na^+-coupled glutamate transporter isoforms described by: Kanai and Hediger 1992, Pines et al., 1992, Storck et al., 1992, Fairman et al., 1995, Kanai et al., 1993 and Kanai et al., 1995. The Na^+-coupled glutamate transporters all exhibit distinct cellular distributions within the periphery and the central nervous system. K_m and V_{max} values obtained for the cerebrocortex were close to the glutamate uptake transporter EAAC1, the neuronal specific transporter cloned by Kanai and Hediger 1992. In contrast, the uptake kinetics for the cerebellum were more akin to both the GLT-1 and EAAT4. As GLT-1 is located on astrocytes, and the presence of astrocyes in this preparation is unlikely [Dunkley et al., 1988], it is more likely that a rat equivalent of the EAAT4 transporter may be responsible. A 92 and 83% reduction in the uptake into cerebrocortical and cerebellar synaptosomes respectively was observed when the temperature was reduced to 0°C, representing a low non-specific uptake.

The addition of L-trans-PDC to the cerebrocortical and cerebellar synaptosomes produced a dose dependent inhibition of [^{3}H]L-glutamate uptake, with maximum inhibition achieved at 100μM of 89 and 82% respectively. The non-specific uptake for cerebrocortical and cerebellar synaptosomes, from this data, was calculated to be 11 and 18% respectively. These values are in agreement with studies at 0°C. The inhibitions of [^{3}H]L-glutamate uptake by L-trans-PDC achieved in this report are consistent with a publication by Robinson et al., 1993, reporting that the potency of L-glutamate to kill cells in primary

hippocampal cultures was increased five-fold upon addition of 100μM L-trans-PDC. In the same study D-AP_5 (NMDA receptor antagonist) almost abolished the toxicity caused by the addition of both L-glutamate and PDC, implicating excessive activation of NMDA receptors in glutamate toxicity. Glutamate uptake inhibitors; L-trans-pyrrolidine-2,4-dicarboxylate (L-trans-PDC) and DL-threo-β-hydroxyaspartate (THA) have been reported to inhibit glutamate uptake leading to an accumulation of endogenous extracellular glutamate in rat neuronal-glial cell cultures [Velasco et al., 1996]. This inhibition correlates in a time-dependent manner with neuronal damage and mimics the toxicity of a short exposure to exogenous glutamate.

Neither anaesthetic agents (intravenous and inhalation) (with the exception of propofol) nor morphine at clinically relevant concentrations, affected the uptake of [^{3}H]L-glutamate under basal conditions into cerebrocortical or cerebellar synaptosomes. Propofol at 30μM in cerebrocortical synaptosomes produced a significant inhibition of the uptake of [^{3}H]L-glutamate. As the brain concentration of propofol can be 8.5 times that in plasma [Shyr et al.,1995], and the plasma concentration is ~1.05μM [Frenkel et al., 1993], this inhibition of [^{3}H]L-glutamate uptake may be within a clinically achievable concentration although the effects would be small. The accumulation of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes under basal conditions was approximately 53 and 65pmol/mg of protein respectively, and under depolarising conditions approximately 14 and 28pmol/mg of protein respectively, but in this thesis have been normalised to 100% for comparison. The reason for these differences may be that under depolarising conditions the values achieved were not carrier mediated uptake but merely a passive exchange of the radiolabel.

Isoflurane was reported to produce a dose-dependent inhibition of the high affinity uptake of [^{3}H]L-glutamate into cerebrocortical synaptosomes by Larsen et al., 1997. However, the reasons for the difference at this stage remains unclear. There are very few detailed studies examining the interaction of anaesthetic agents with glutamate uptake, however studies examining their effect on glutamate release are more abundant (see also Chapter 6, Section6.4, page 131). In the anaesthetised rabbit (5% halothane in O_2), neither temperature reduction nor pentobarbital, isoflurane or propofol, affected the in vivo glutamate concentrations measured in the dorsal hippocampus by microdialysis [Illievich

et al., 1994]. Isoflurane was reported to reduce both the Ca^{2+}-independent and -dependent release of glutamate in rat hippocampal slices dissected after pentobarbital anaesthesia [Larsen et al., 1994]. In addition, Schlame et al., 1995, have reported that the 4-AP-evoked release of glutamate from rat cerebrocortical synaptosomes was dose dependently reduced by isoflurane, halothane, enflurane and pentobarbital. Bickler et al., 1995, reported that halothane, enflurane, thiopentone but not propofol (at clinically relevant concentrations), decreased the hypoxic-induced extrasynaptic accumulation of glutamate from rat cortical slices.

Opioids represent an important class of therapeutic agents used in the pre-, intra- and post-operative periods, where presumably their mechanism of action is via a reduction in neurotransmission (see also Chapter 5, Section 5.4, page 107) [Childers 1993]. Yet the effect of opioids on neurotransmitter uptake receives little attention. In rat cerebrocortical slices Bradford et al., 1986 reported that μ and κ opioids inhibited veratrine-stimulated release of glutamate, morphine (10μM) inhibition was 116%. Hayward et al., 1995, have shown that the κ agonist enadoline is effective in reducing cerebral damage due to transient ischaemic episodes in the cerebral cortex of rats. However neither of these studies examined the uptake process. The data presented in this chapter suggests that the inhibition of glutamate release observed in the above studies did not involve the inhibition of glutamate uptake.

In summary, the uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes was time-, Na^+, temperature-, concentration-dependent and L-trans-PDC sensitive. The range of anaesthetic and analgesic agents studied failed to affect the uptake at clinically relevant concentrations, therefore any effects of these agents on glutamate release (described in the remainder of this thesis) are likely to directly involve the release process.

Chapter 4. Development of a Method for the Measurement of Glutamate Release from Rat Brain Tissue.

4.1. Introduction

Brain preparations including synaptosomes, brain slices and tissue homogenates have been used for many years as model systems for the study of the uptake, storage and release of neurotransmitters in vitro [Griffiths & Norman 1993].

In 1972 De Belleroche and Bradford compared the incorporation of [U-^{14}C]glucose into glutamate, GABA and aspartate. In response to synaptosomal depolarisation using both electrical and 56mM K^+-stimulation of synaptosomes, a statistically significant increase in release of the neurotransmitter release was seen. Released amino acids were quantified following separation using ion exchange chromatography. The removal of Ca^{2+} from and addition of EGTA to the incubation medium, produced an inhibition of K^+-evoked glutamate, GABA and aspartate release without altering the basal release confirming Ca^{2+} regulated exocytotic release

In order to minimise the effects of re-uptake (see Chapter 3), Raiteri et al., 1974 devised a superfusion chamber allowing an accurate and continuous measurement of the release of GABA and noradrenaline from radio-labelled synaptosomes prepared from rat cerebrum. The release of GABA and noradrenaline in superfused tissue was greater than that seen with static incubation protocols [Baldessarini 1971; Ng et al., 1972]. Clearly dynamic release protocols have several advantages over previous static incubation protocols. These include, analysis of the time course of release, introduction of different buffers and minimisation of re-uptake.

Collard et al., 1991 examined the effects of perfusion rate (1, 2, 4, 10 and 20ml/min) on glutamate release from rat cortical synaptosomes prelabelled with [^{3}H]glutamate. They showed that a release of 2.489% of tissue glutamate/min was achieved at 10ml/min

following stimulation with 15mM K^+, compared to a release of 0.308% of tissue glutamate/min at 1ml/min.

Waldmeier et al., 1993, reported that the release of endogenous glutamate from perfused rat cerebrocortical slices evoked by electrical field stimulation at low frequencies was not measurable, but in the presence of L-trans-PDC (a glutamate uptake inhibitor) glutamate release could be quantified. 15mM K^+-evoked glutamate release in the presence of L-trans-PDC was reduced by 25-50% in comparison with no L-trans-PDC. The reasons for these differences are unclear.

A novel method for the measurement of endogenous glutamate release from guinea pig cerebrocortical synaptosomes was reported by Nicholls and Sihra 1986. This "continuous enzymatic assay" was based on the oxidation of the released glutamate to 2-oxoglutarate accompanied by the reduction of NAD^+ to NADH, which fluoresces at 366nm excitation (430nm emission). They concluded that 15-20% of the total synaptosomal glutamate can be rapidly released by 30mM K^+ in a Ca^{2+}- dependent manner. This method has recently been used by Cousin et al., 1995 in cultured cerebellar granule cells where 50mM K^+ was shown to evoke exocytotic release.

Aims

In the previous chapter the effects of a range of commonly used anaesthetic/analgesic agents on the uptake of glutamate into cortical and cerebellar synaptosomes was reported. The next phase of this project was to establish a reliable method for the measurement of glutamate release from rat brain tissue. This chapter describes the development of a method for the measurement of glutamate release.

4.2. Methods

Cerebrocortical and Cerebellar Synaptosomal Glutamate Release

From previous reports, a method widely used in synaptosomes to study neurotransmitter release involved: loading the brain preparation with a radioactive label, washing the tissue, stimulating neurotransmitter release and separating the desired neurotransmitter from other substances possibly co-released. We have used this method as a basis for further studies in this chapter.

1. [^{3}H]L-glutamate Loading Protocols

Cerebrocortical and cerebellar synaptosomes were prepared as in Chapter 2, Section 2.3, page 43. Synaptosomes were centrifuged for 10min at 4°C at 20,000g. 100nM [^{3}H]L-glutamate in 1ml HEPES buffer was added to the synaptosomal pellet for 5 or 15min.

2. Washing Synaptosomes

After "loading", the synaptosomes were re-centrifuged at 20,000g for 10min at 4°C. The synaptosomes were "washed" by the addition of 20ml HEPES buffer for 5, 15 or 30min after which they were re-centrifuged as before. The washing procedure was repeated three times and the final synaptosomal pellet was resuspended in 5-6ml of HEPES buffer.

3. Release of Glutamate from Synaptosomes

The release of glutamate from cortical and cerebellar synaptosomes at 37°C was performed in HEPES buffer under static conditions and carried out in 500 or 1000µl volumes. The different methods of release will be described later in this chapter.

4. Extraction of Glutamate

At the end of the incubation period the reactions were terminated by either rapid vacuum filtration onto Whatman GF/B filters (washed with 0.5ml ice-cold HEPES buffer when stated) using a Millipore cell harvester, or rapid centrifugation for 2min at 1,000rpm. If the reaction was terminated by rapid vacuum filtration, the filters were extracted overnight with Optiphase "Safe" as a scintillant and the activity quantified by liquid scintillation spectroscopy using a Packard 1900 TR Liquid Scintillation Analyser. Any [^{3}H]L-

glutamate present in the filtrate was also assayed. If the reaction was terminated by rapid centrifugation, the supernatant radioactivity was measured by liquid scintillation spectroscopy using Optiphase High Safe 3 as a scintillant. The synaptosomal pellet was digested by the addition of 250µl of 1M NaOH for 30min, after which the radioactivity was quantified.

5. Separation of Released Glutamate

If glutamate dehydrogenase is present within the synaptosomes, any glutamate released will be catalytically oxidised to 2-oxoglutarate. Thus it was essential to confirm that the tritiated substance released and subsequently measured was glutamate. This was confirmed by High Pressure Liquid Chromatography (HPLC). A 250µl sample of the tritiated substance was injected into a 250µl loop, via a Rheodyne injection port using a 500µl glass Hamilton syringe and separated by a 15cm hypersil ODS C18 (particle size 3µm) HPLC column. The system was maintained by a peristaltic pump at a rate of 1.5ml/min, with a filtered solution of 80% 20mM sodium acetate, 19% HPLC grade methanol and 1% tetrahydrofuran. Fractions were collected every 15secs, and each fraction was assayed for radioactivity by liquid scintillation spectroscopy.

4.3. Individual Release Protocols (1-5), Results (1-5) and Conclusions (1-5)

Release Protocol 1 (Synaptosomes)

Cerebrocortical and cerebellar synaptosomes were loaded and washed for 15 and 5min respectively. From 5mls of resuspended cerebrocortical and cerebellar synaptosomes, 480µl were depolarised with 46mM K^+ (20µl from a 1.15M stock) for 1, 2, 5 and 10min in a final volume of 0.5ml. The extraction of released [^{3}H]L-glutamate was performed by rapid vacuum filtration. During all the experiments, an equivalent amount of NaCl was removed when depolarising with KCl to maintain tonicity, and glutamate release was measured as a % of the total amount of glutamate present.

Results from Protocol 1

Glutamate release from cerebrocortical synaptosomes rose steadily from a basal of 15.03% to 23.77% at 2min stimulation, then increased to 29.51% at 10min (Fig 4.1). In cerebellar synaptosomes, glutamate release rose from a basal of 10.31% to 19.34% at 5min and 17.35% at 10min (Fig 4.1).

Comments from Protocol 1

Basal glutamate release obtained using protocol 1 was high in both the cerebrocortex and cerebellum, yet the changes from basal to depolarised release were small. This might have been due to small volumes of synaptosomes used in the experiment and the difficulty in harvesting/washing using the Millipore system. The volume of synaptosomes was increased to 980µl and the depolarisation was for 10min, as this produced the largest change in glutamate release compared to basal within cerebrocortical synaptosomes. However, it should be considered that high K^+ depolarisation for long periods has been shown to reverse the glutamate uptake transporter (see Chapter 3).

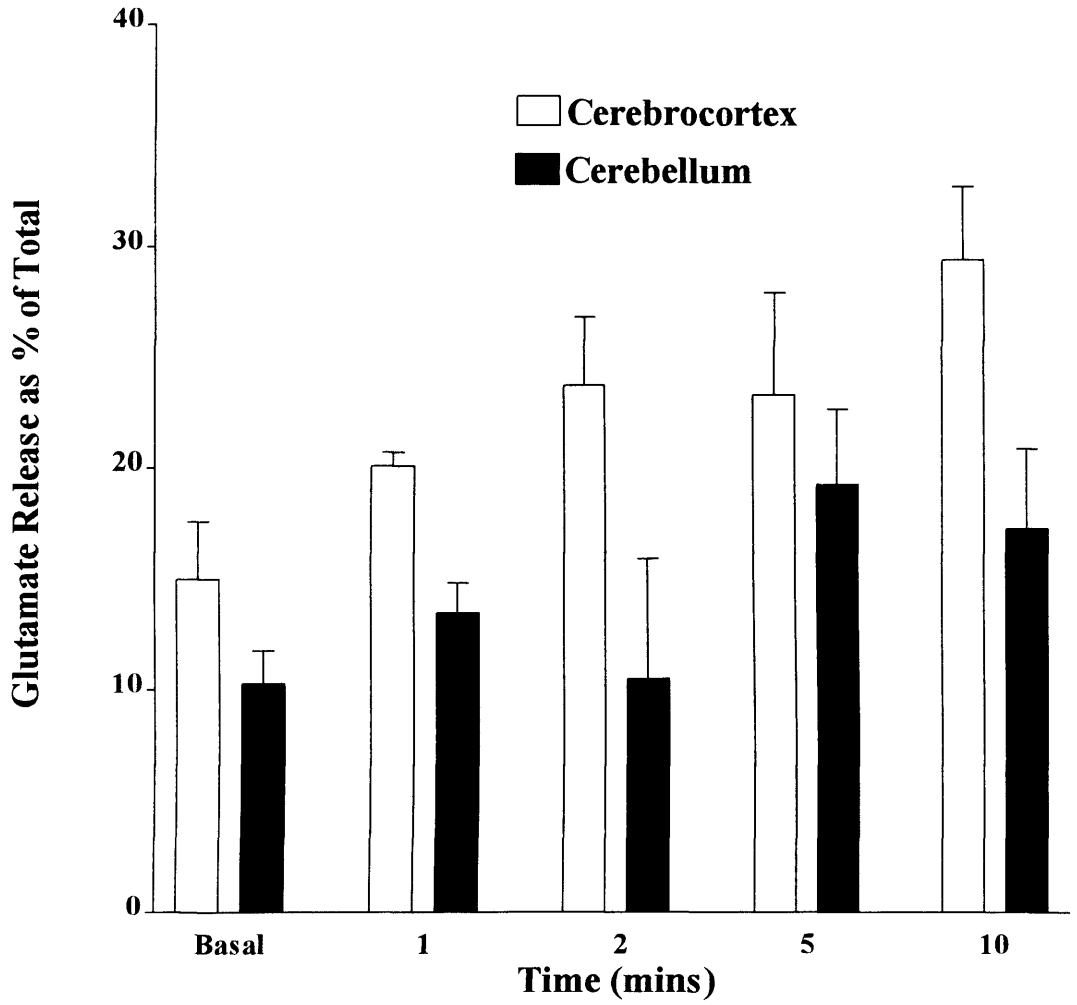


Fig 4.1. The effect of increasing time intervals for 46mM K^+ stimulation of cerebrocortical and cerebellar synaptosomes. Kruskal Wallis test was not performed due to low sample size. Data are mean±sem (n=3).

Release Protocol 2 (Synaptosomes)

Cerebrocortical and cerebellar synaptosomes were loaded and washed for 15 and 5min respectively. From 5mls of resuspended cerebrocortical and cerebellar synaptosomes, 980µl were incubated with 46mM K^+ for 10min in a final volume of 1ml. The extraction of the released glutamate was performed by rapid vacuum filtration. Additional confirmation of the released substance as glutamate was performed by HPLC. In total 3 samples were injected onto the column: a standard concentration of [^{3}H]glutamate and supernatants from basal and depolarised cerebrocortical synaptosomes.

Results from Protocol 2

Glutamate release from cerebrocortical and cerebellar synaptosomes increased by 75 and 98% respectively above basal upon the addition of 46mM K^+ for 10min. Basal release from cerebrocortical synaptosomes was 36.2% of the total glutamate present in the synaptosomes, in the cerebellum it was 22.96% (Fig 4.2). When basal and K^+ stimulated effluent from cerebrocortical synaptosomes were injected onto the HPLC column, they co-eluted with a [^{3}H]glutamate standard, confirming the radiolabelled substance measured as glutamate (Fig 4.3).

Comments from Protocol 2

Using protocol 2, basal glutamate release remained high (36% in the cerebrocortex and 23% in the cerebellum), but following depolarisation a 75 and 98% increase in depolarised glutamate release was achieved, compared to basal glutamate release in cerebrocortical and cerebellar synaptosomes respectively. Based on protocols 1 and 2, increasing the volume of synaptosomes did not decrease the basal release of glutamate from synaptosomes, but it did increase the depolarised release. As volume has been tested as a variable, varying the washing time was the next variable to be tested. The cerebrocortex of the rat is considerably bigger in mass than that of the cerebellum, therefore we chose to study cerebrocortical synaptosomes and concentrate on the manipulation of the methodological procedure in this tissue.

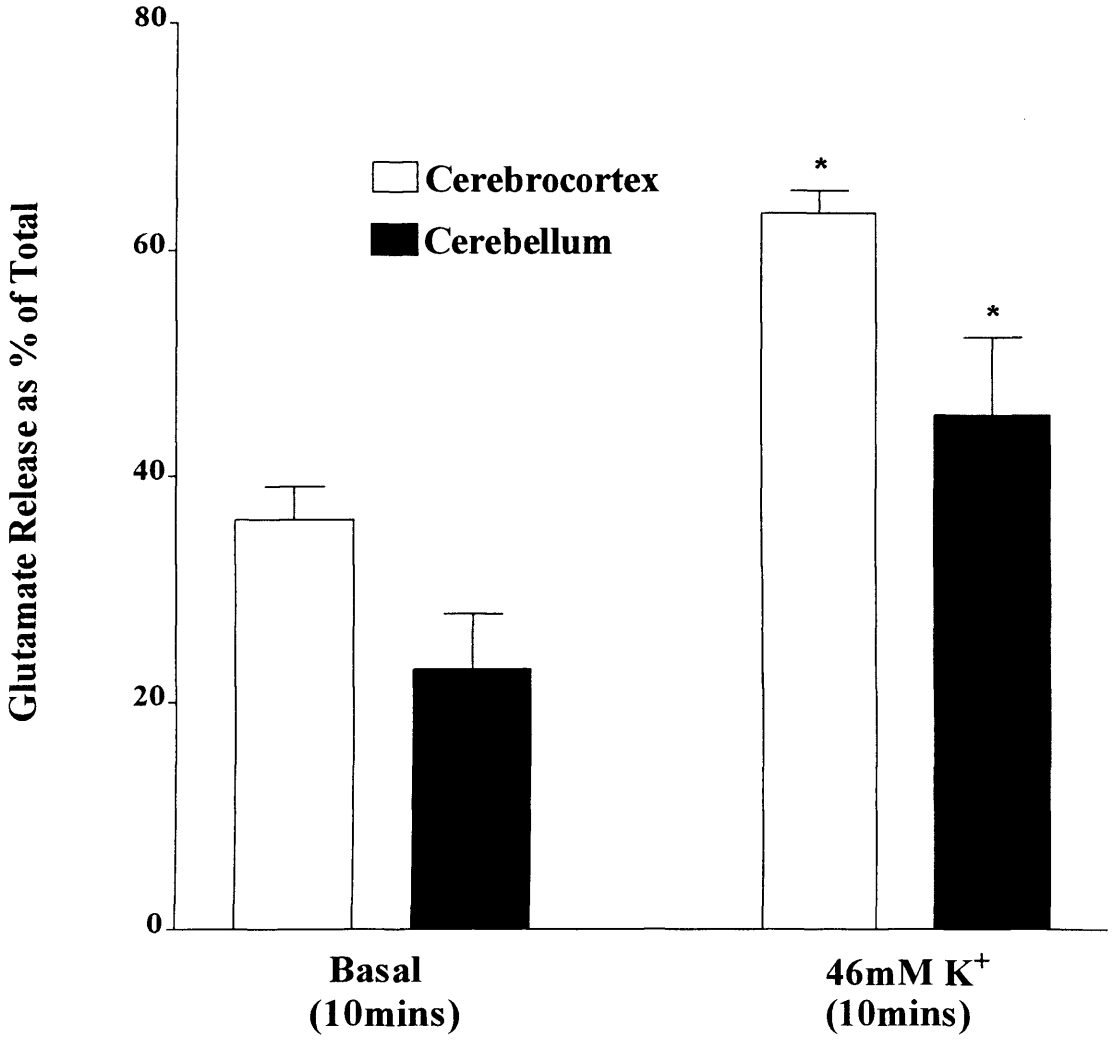


Fig 4.2. A 10min pulse of 46mM K^+ significantly increases the release of glutamate from rat cerebrocortical and cerebellar synaptosomes compared to basal. Basal glutamate release is also measured at 10min. Data are mean±sem (n=5) and * illustrates statistical significance compared with corresponding basal.

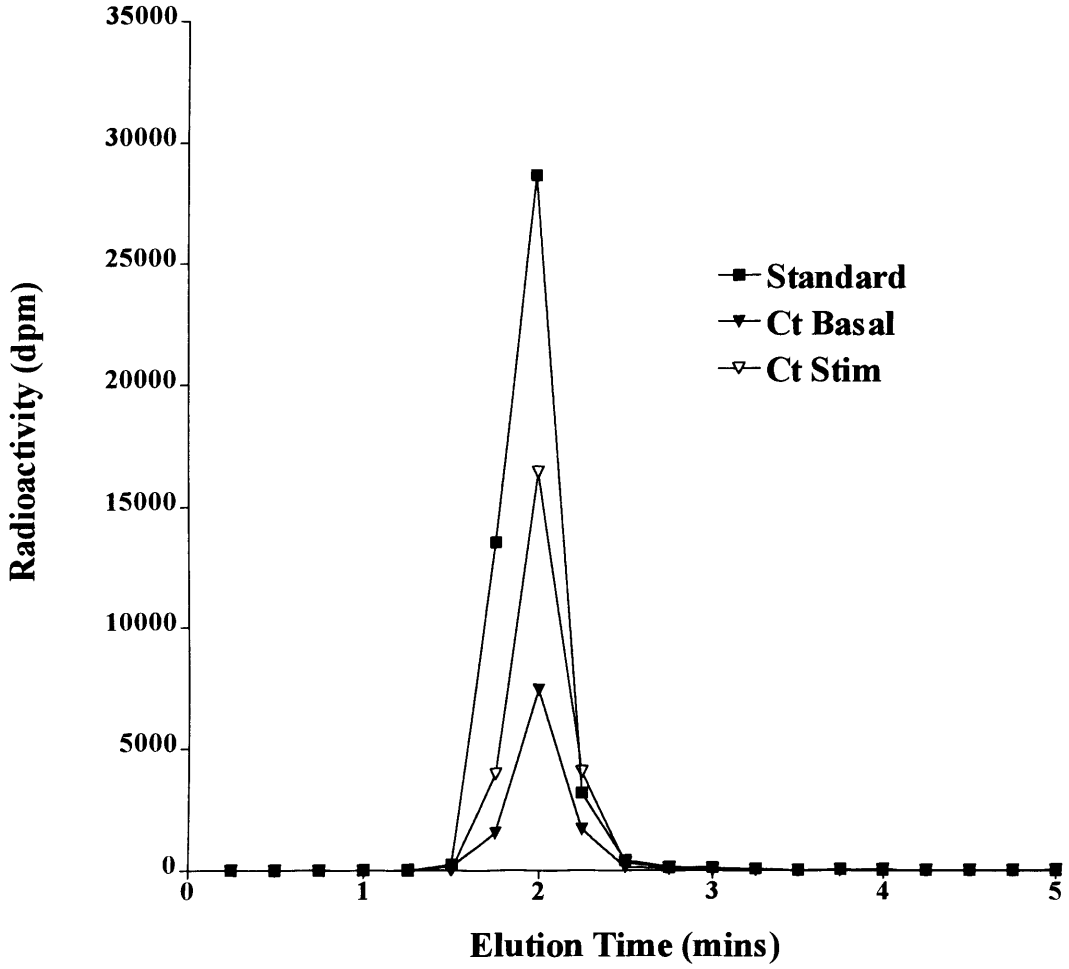


Fig 4.3. Samples from basal and depolarised synaptosomes co-eluted with [^{3}H]L-glutamate standard when separated by HPLC and quantified by liquid scintillation spectroscopy. Ct Basal: basal sample. Ct Stim: sample of stimulated cerebrocortical synaptosomes.

Release Protocol 3 (Synaptosomes)

Cerebrocortical synaptosomes were loaded for 15 and washed for either 15 or 30min. From 5mls of resuspended cerebrocortical synaptosomes in HEPES buffer, 480µl alliquots were pre-incubated at 37°C for 5min prior to depolarisation with 46mM K^+ for 1, 2, 3, 4 and 5min in a final volume of 0.5ml. Basal glutamate release was also examined at the same time intervals. The extraction of the released glutamate was performed by rapid vacuum filtration.

Results from Protocol 3

After a washing time of 15min, evoked release at 3min depolarisation amounted to an enhancement of 95% over basal. At 1, 2, 4 and 5min the enhancement was lower. After a washing time of 30min increases in the release of glutamate following depolarisation were smaller, but it should be noted that this is data from a single experiment (Fig 4.4).

Comments from Protocol 3

Basal release from cerebrocortical synaptosomes washed for 15min remained high, and a 3min pulse of 46mM K^+ produced an increase of 95%. Increasing the washing time to 30min generally reduced the basal glutamate release, however the change in glutamate release from basal to depolarised remained small. Previous reports have used a higher concentration of K^+ to depolarise synaptosomes [De Belleroche and Bradford, 1972 & 1977], therefore the K^+ concentration was increased.

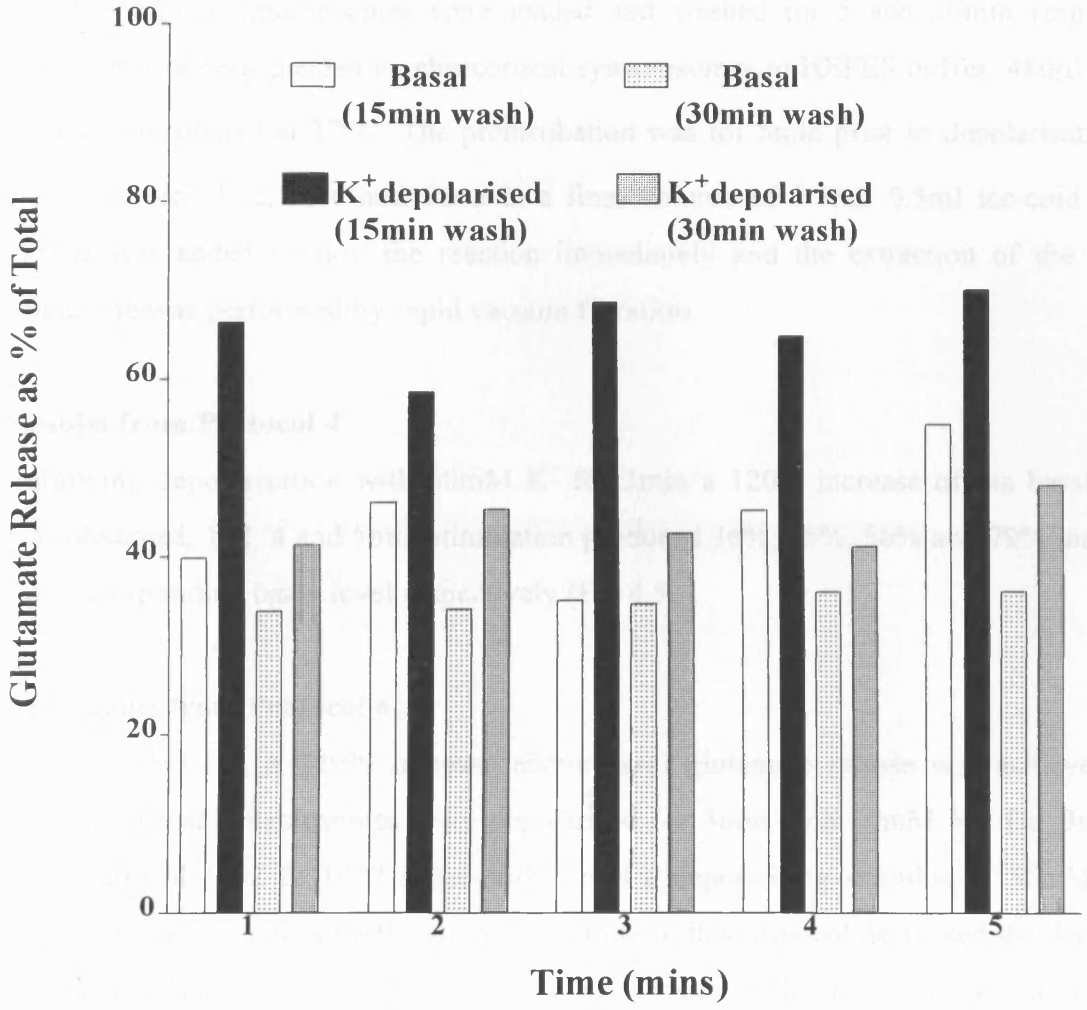


Fig 4.4. The effect of increasing time intervals for 46mM K^+ stimulation of cerebrocortical synaptosomes. This graph is from one experiment.

Release Protocol 4 (Synaptosomes)

Cerebrocortical synaptosomes were loaded and washed for 5 and 30min respectively. From 6mls of resuspended cerebrocortical synaptosomes in HEPES buffer, 480µl aliquots were pre-incubated at 37°C. The preincubation was for 5min prior to depolarisation with 60mM K^+ for 1, 2, 3, 4 and 5min in a final volume of 0.5ml. 0.5ml ice-cold HEPES Buffer was added to stop the reaction immediately and the extraction of the released glutamate was performed by rapid vacuum filtration.

Results from Protocol 4

Following depolarisation with 60mM K^+ for 3min a 120% increase of the basal release was observed. 1, 2, 4 and 5min stimulation produced 36%, 95%, 56% and 79% increase in the corresponding basal level respectively (Fig 4.5).

Comments from Protocol 4

Using protocol 4, a 120% increase above basal glutamate release was achieved when cerebrocortical synaptosomes were depolarised for 3min with 60mM K^+. De Belleroche and Bradford 1972 & 1977 successfully used a depolarising stimulus of 56mM K^+ for 10min, however increasing the stimulation time in this protocol decreased the depolarised glutamate release. Basal glutamate levels were lower in this protocol compared to other protocols used, amounting to 11% of the total glutamate present in the synaptosomes at 3min. The basal release of glutamate remains unacceptable, despite the increase from 11 to 24% when the synaptosomes were depolarised.

Using a static incubation procedure, the problem of re-uptake has not been considered. In the previous chapter, maximum glutamate uptake into cerebrocortical synaptosomes was achieved at 1min and glutamate uptake was inhibited by L-trans-PDC in a dose dependent manner with IC_{50} values of 13.24 and 3.94µM respectively (see Chapter 3, Section 3.3, pages 54-62). Therefore, the next step was to examine the effects of L-trans-PDC on basal and K^+ evoked glutamate release.

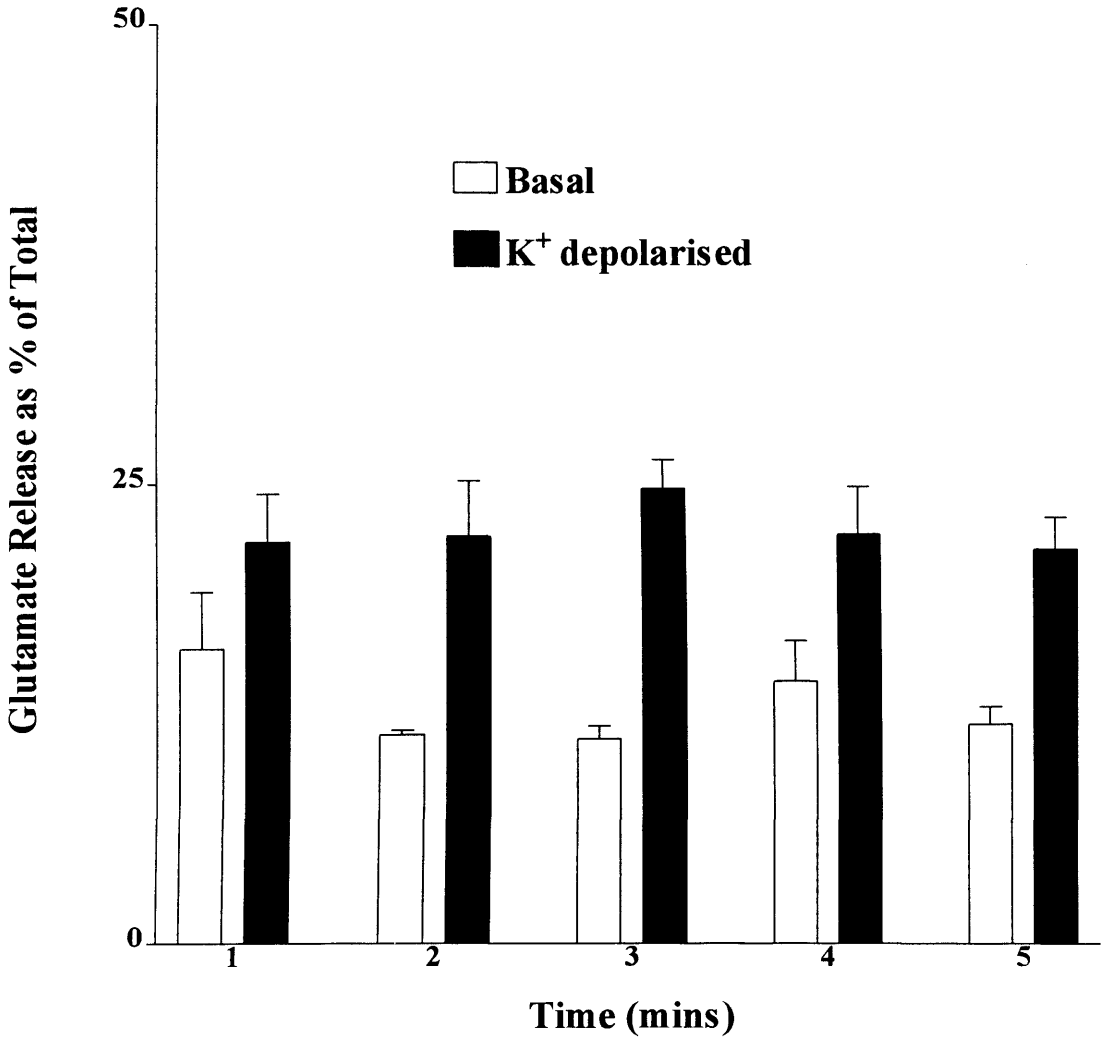


Fig 4.5. The effect of increasing time intervals for 60mM K^+ stimulation of cerebrocortical synaptosomes. Kruskal Wallis test was not performed due to low sample size. Data are mean±sem (n=3).

Release Protocol 5 (Synaptosomes)

Cerebrocortical synaptosomes were loaded and washed for 5 and 30min respectively. From 6mls of resuspended cerebrocortical synaptosomes in HEPES buffer, 480µl aliquots were pre-incubated at 22°C for 2hrs. The temperature during the pre-incubation period of 2hrs was maintained at 22°C as this was the temperature that Waldmeier et al., 1993 used for the measurement of glutamate release in the presence of L-trans-PDC. 100µM L-trans-PDC was then added to the synaptosomes and incubated for 10min at 37°C, prior to depolarisation with 60mM K^+ for 1, 2, 3, 4 and 5min in a final volume of 0.5ml. The extraction of released glutamate was performed by rapid vacuum filtration, and on this occasion the protocol was repeated and the released glutamate was extracted by centrifugation.

Results from Protocol 5

At 1 and 2min 60mM K^+-evoked glutamate release extracted by filtration was lower than basal release, and at 3, 4 and 5min the evoked release was enhanced by 17, 63 and 18% respectively compared to basal. When the reaction was terminated by rapid centrifugation, both basal and depolarised release increased. At 1, 2, 3, 4 and 5min an increase of 22, 29, 62, 34 and 35% compared to basal was observed (Fig 4.6).

Comments from Protocol 5

L-trans-PDC was used to inhibit glutamate uptake into cerebrocortical synaptosomes, and enhance the K^+-evoked release. This did not occur.

A comparison of filtration and centrifugation extraction processes were performed. Centrifugation produced an increase in basal glutamate release compared to filtration and the change in released glutamate compared from basal to depolarised remained small as in filtration. The increase in basal release following centrifugation may be due to crushing of the synaptosomes, resulting in the release of glutamate present within the synaptosomes.

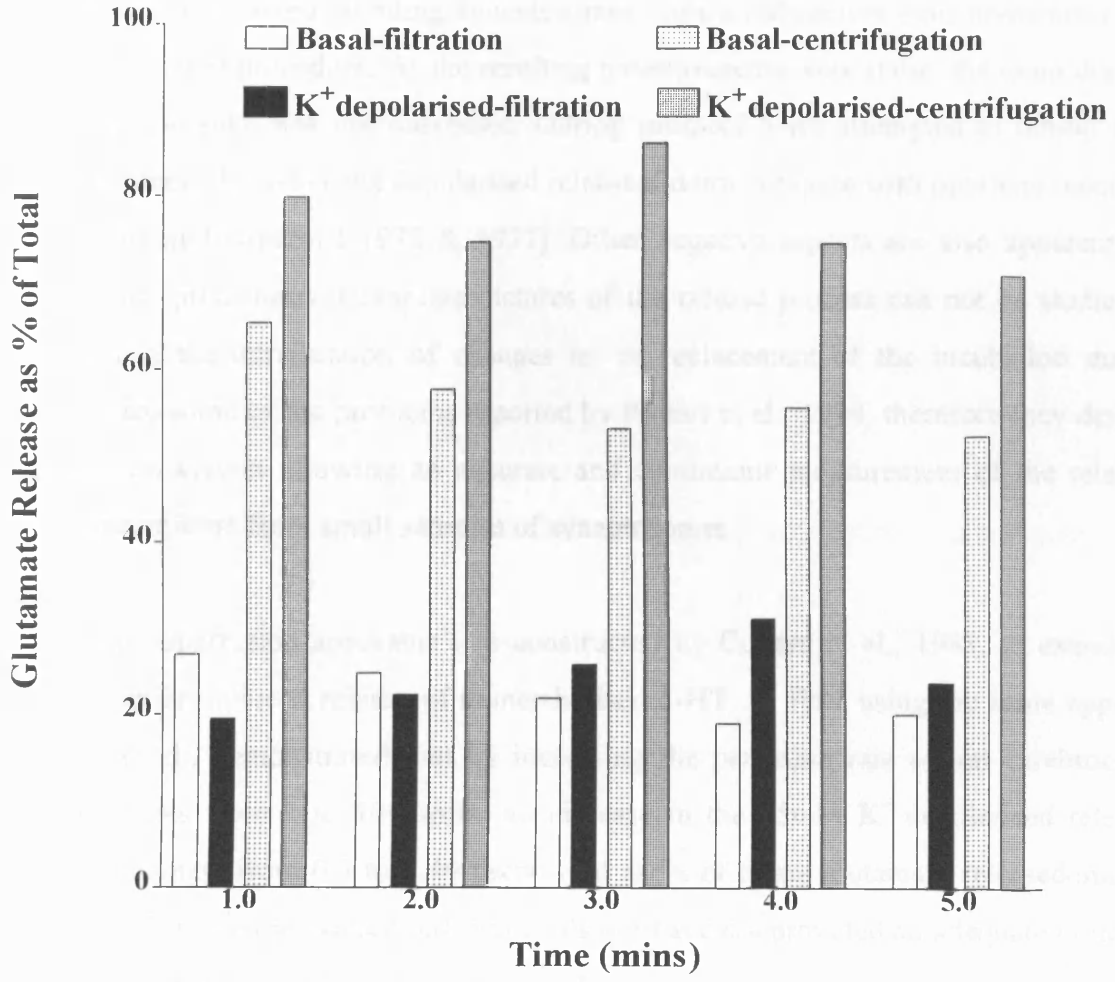


Fig 4.6. The effect of increasing time intervals for 60mM K^+ stimulation of cerebrocortical synaptosomes in the presence of 100μM L-trans-PDC. This data is from one experiment.

4.4. Conclusion from Protocols 1-5

Protocols 1-5 involved labelling synaptosomes with a radioactive neurotransmitter and a lengthy washing procedure. As the resulting measurements were static, the main drawback was that re-uptake was not prevented. During protocol 5 we attempted to inhibit the re-uptake process, however the depolarised release did not compare with previous reports [De Belleroche and Bradford 1972 & 1977]. Other negative aspects are also apparent when using static procedures. Complete pictures of the release process can not be studied, and neither can the introduction of changes to, or replacement of the incubation medium. These were some of the problems reported by Raiteri et al., 1974, therefore they devised a superfusion system allowing an accurate and continuous measurement of the release of neurotransmitters from small samples of synaptosomes.

A similar superfusion apparatus was constructed by Collard et al., 1981, to examine the depolarisation-induced release of unmetabolised 5-HT. In 1991 using the same apparatus, Collard et al., demonstrated that by increasing the perfusion rate on rat cerebrocortical synaptosomes from 1 to 10ml/min, an increase in the 15mM K^+ depolarised release of [^{3}H]L-glutamate from 0.3 to 2.49 (expressed as % of tissue glutamate released/min) was produced. Despite the evaluations, protocols 1-5 have not provided an adequate protocol to measure basal and depolarised glutamate release.

Nicholls and Sihra 1986, published a method of measuring glutamate release directly from synaptosomes using a continuous enzymatic assay. Guinea pig cerebrocortical synaptosomes were incubated in a medium containing glutamate dehydrogenase and NAD^+. Any released glutamate would be catalytically oxidised to 2-oxoglutarate and the NAD^+ reduced to NADH, producing an increase in fluorescence measured at specific excitation and emission wavelengths for NADH. An attempt was made to reproduce this method using rat cerebrocortical synaptosomes. However, when a depolarising stimuli of 46mM K^+ was applied, an increase in fluorescence was not observed.

Previous experiments in our laboratory involved the perfusion of SH-SY5Y human neuroblastoma cells. The perfusion chamber was constructed from a 2ml syringe barrel, packed with glass wool, and the cells were pipetted into the chamber on top of the glass

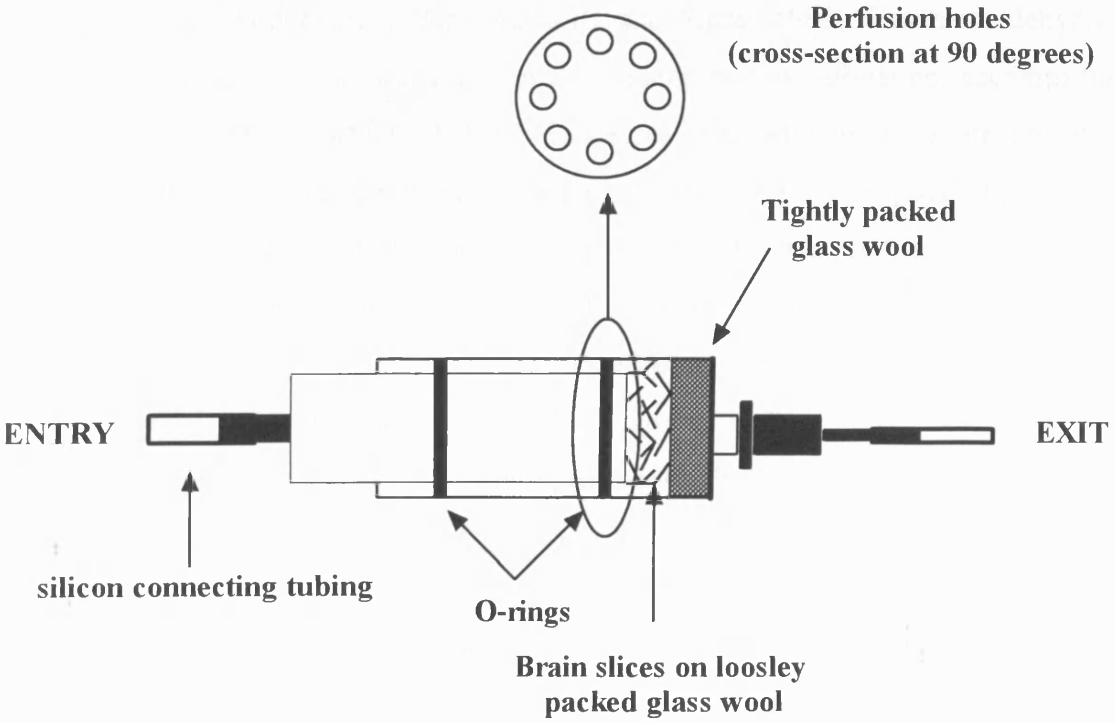
wool. Inserted into the chamber was a perspex flow diffuser, to produce even perfusion. This system was used to measure noradrenaline release and to examine the effects of anaesthetic agents on release [Atcheson et al., 1994]. An evaluation of the use of this system for measurement of glutamate release was performed. As preliminary perfusion studies with synaptosomes revealed that they were present in the effluent, perfused cerebrocortical slices were chosen.

4.5. Cerebrocortical Slice Glutamate Release Methods

Perfusion Chamber

Cerebrocortical slices were prepared from female Wistar rats as previously described in Chapter 2, Section 2.4, page 43. The perfusion chamber consisted of a 2ml syringe barrel cut at approximately 1.75cm and packed at the needle end with approximately 0.5cm glass wool. Approximately 1ml of gravity packed slices (protein not determined) were pipetted into the barrel, followed by a greased diffuser. The chamber was sealed around the diffuser by 2 O-rings (Fig 4.7). The perfusate entered via a Bio Rad Econo peristaltic pump which provided a continuous, steady flow to the cerebrocortical slices at the diffuser end, and exited at the needle end of the syringe. The eluate was collected using a Glison FC205 fraction collector.

Fig 4.7 The perfusion chamber as described by Atcheson et al., 1994. This figure was kindly provided by Dr. Robert Wilcox, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester.



Release Protocol

The release of glutamate from cerebrocortical slices was performed in oxygenated (95% O_2/5% CO_2) bicarbonate buffer. Prior to the insertion of the slices into the perfusion chamber, they were agitated in a shaking water bath maintained at 37°C for 40min. As stated previously, approximately 1ml of gravity packed slices was pipetted into a perfusion chamber (Fig 4.7). The slices were perfused at a rate of 1ml/min for 60min prior to the collection of 2ml fractions, for the estimation of glutamate concentrations. To stimulate the slices 46mM K^+ was applied for 2min. This was the concentration and stimulation time used by Cousin et al., 1995, who measured glutamate release in cultured cerebellar granule cells. Following 6min of perfusion, a 2min pulse of 46mM K^+ was applied. As this was the first depolarising stimulus applied to the slices, it was termed S_1. The slices were perfused for a further 30min prior to a second application of 46mM K^+, this was termed S_2. After S_2, 8min of fractions were collected after which the experiment was terminated. S_1 acted as an internal control for tissue integrity.

Measurement of Glutamate Release

The fractions collected were analysed for glutamate release using an adapted version of the fluorescence method employed by Nicholls and Sihra, 1986. Glutamate dehydrogenase (GDH) catalytically reduces any glutamate present to 2-oxo-glutarate, accompanied by a reduction of NAD^+ to NADH. NADH fluoresces at an excitation wavelength of 366nm, and emits at 430nm. However as NADH undergoes reoxidation [Nicholls et al., 1987], $NADP^+$ was used in place of NAD^+ forming NADPH when reduced (Fig 4.8). NADPH fluoresces at the same wavelengths as NADH. The time to completion was assessed by monitoring the change in fluorescence intensity at 37°C with respect to time, using a Perkin-Elmer LS50B spectroflurimeter. As illustrated in Fig 4.9, the reaction plateaued at 5min and remained constant until 10min, therefore all samples were incubated for 10min at 37°C. Prior to the start of each experiment, a calibration curve was constructed (Fig 4.10), in which 10µl of known concentrations of glutamate were incubated with 5µl NADP (final concentration of 1mM) and 15µl GDH (final concentration of 30U) made up to a final volume of 0.5ml using bicarbonate buffer. From a 2ml fraction collected, 480µl perfusate was incubated with 5µl NADP (1mM), and 15µl of GDH (30 Units) for 10min at

37°C. The resulting production of NADPH was compared with a known set of glutamate standards.

Fig 4.8. Reaction for the oxidation of glutamate using GDH.

$$\textbf{Glutamate} + \textbf{NAD}^+ \longrightarrow \textbf{2-oxo-glutarate} + \textbf{NADH} + \textbf{NH}_3^+ \textbf{ (using GDH)}$$

OR

$$\textbf{Glutamate} + \textbf{NADP} \longrightarrow \textbf{2-oxo-glutarate} + \textbf{NADPH} + \textbf{NH}_3^+ \textbf{ (using GDH)}$$

4.6. Results

Fig 4.11 illustrates that 2×2min pulses of 46mM K^+ produced monophasic releases of glutamate. The two pulses of 46mM K^+ (S_1 and S_2) were applied at fractions 4 and 20 respectively, however the increase in glutamate release was not visible until fractions 5 and 6, and 21 and 22 respectively as the dead volume of the tubing was approximately 2.5ml. Fractions 1-24 were initially analysed, however as there was no difference in the release of glutamate between fractions 9-16, only fractions 1-8 and 17-24 were subsequently analysed. Glutamate release is illustrated in Fig 4.11 relative to the mean of the first three basal samples collected. This measurement normalises for differences in protein. The areas under the stimulation curves were calculated to account for the overall effect of the stimulation and not just the peak response. The data was also represented as S_2/S_1 ratios using the formula as described below, where F = fraction number:

$$(F_{20} + F_{21} + F_{22} + F_{23} + F_{24}) - ((F_{17} + F_{18} + F_{19}) \div 3 \times 5) = S_2$$

$$\div$$

$$(F_4 + F_5 + F_6 + F_7 + F_8) - ((F_1 + F_2 + F_3) \div 3 \times 5) = S_1$$

When areas under the stimulation curves were calculated a mean S_2/S_1 ratio of 1.07±0.08 (75) was obtained.

4.7. Comments

Fig 4.11 clearly shows that when a 2min depolarising pulse of 46mM K^+ is applied to the cerebrocortical slices, an increase in glutamate release is observed. The percentage increases in glutamate release when compared to that of basal, correspond to the 56mM K^+-evoked glutamate release observed by De Belleroche and Bradford 1977, who reported a 2-4 fold increase of glutamate, GABA and aspartate release compared to basal. As this method produced substantial reproducible releases on application of a 46mM K^+ pulse, with the resulting depolarisations of a magnitude similar to previous reports [De Belleroche and Bradford 1972 & 1977], the investigation into the effects of opioids and anaesthetic agents on perfused rat cerebrocortical slices began using this perfusion method.

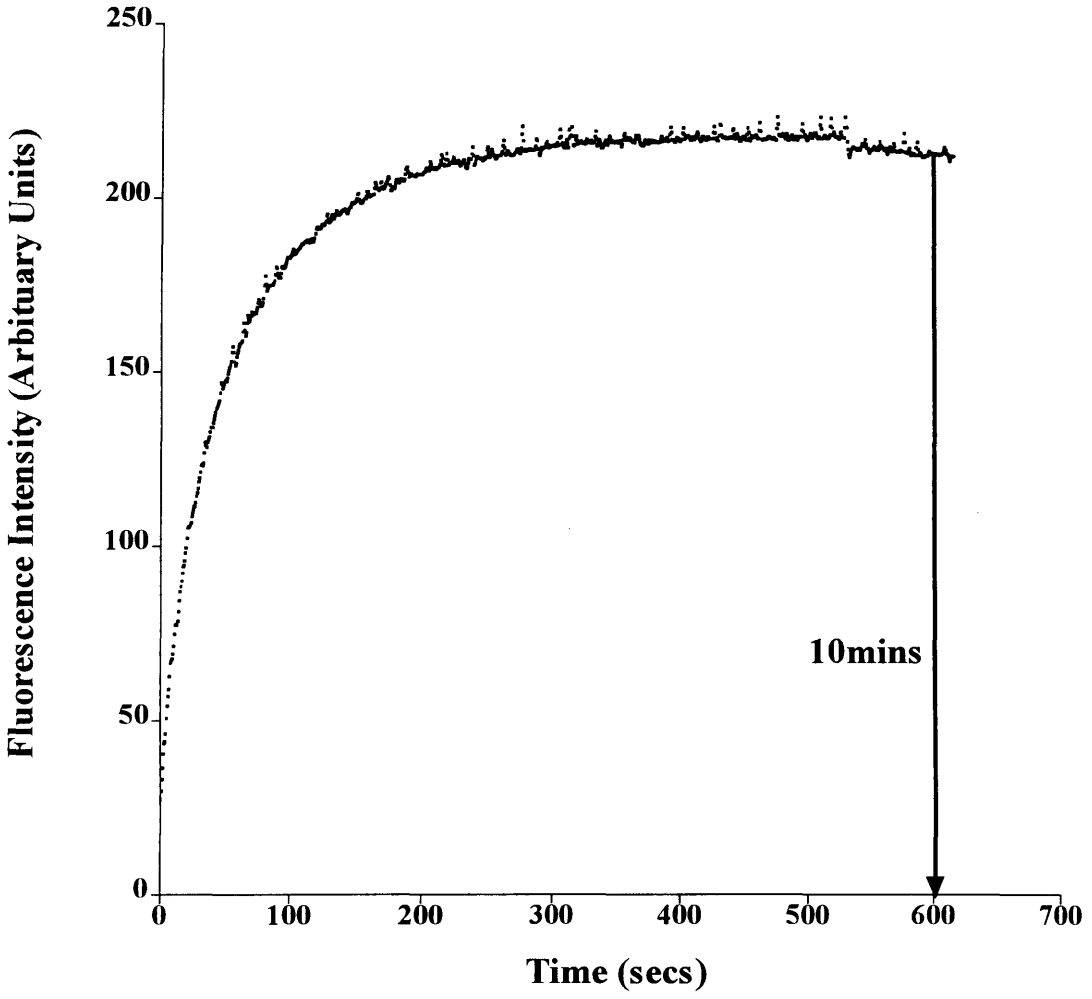


Fig 4.9. Glutamate present has been oxidised to 2-oxo-glutarate accompanied by the reduction of NADP to NADPH by glutamate dehydrogenase. This data is from a typical experiment.

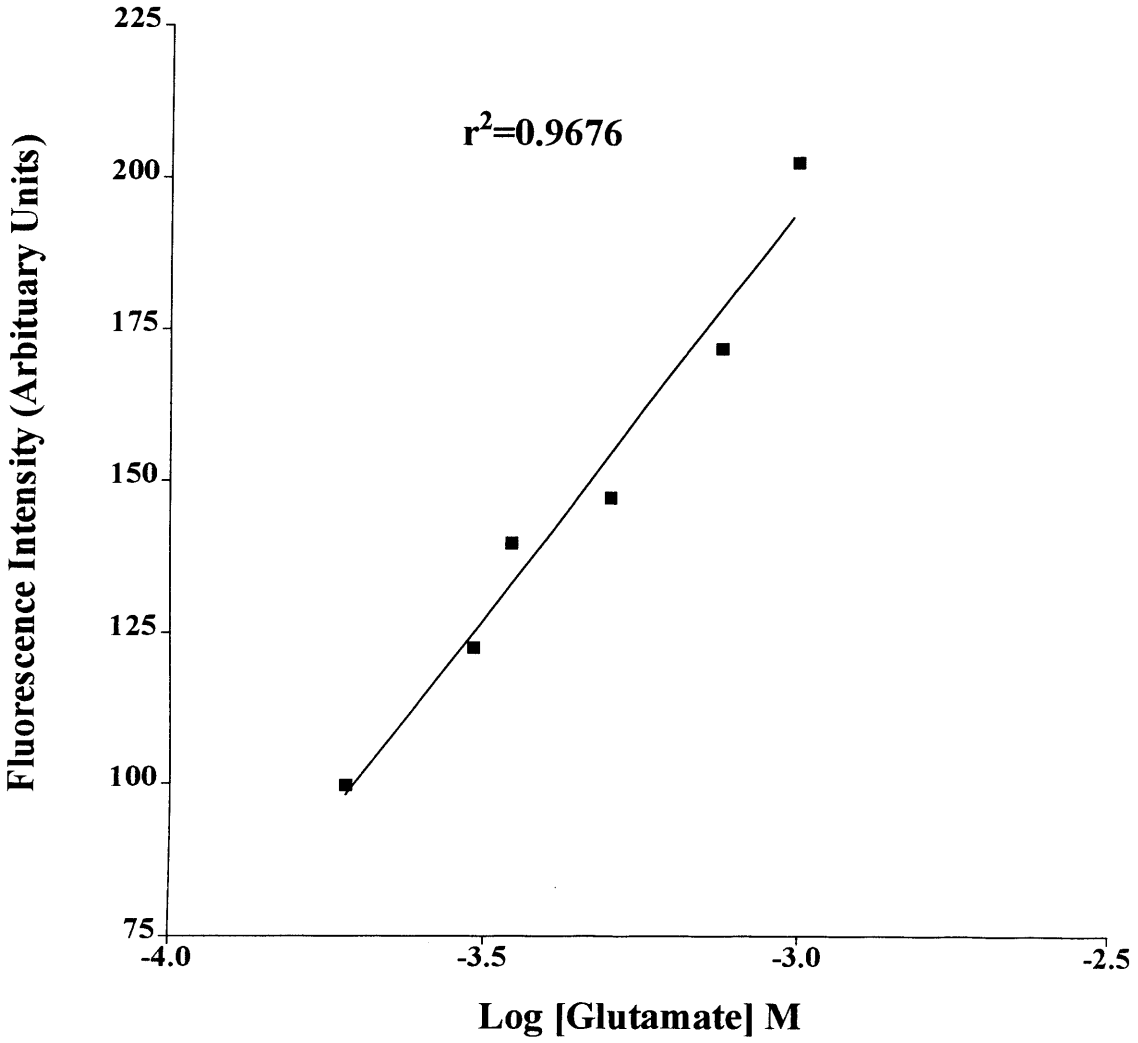


Fig 4.10. Standard curve for the measurement of glutamate concentrations at excitation and emission wavelengths of 366 and 430nm. This data is from a typical experiment.

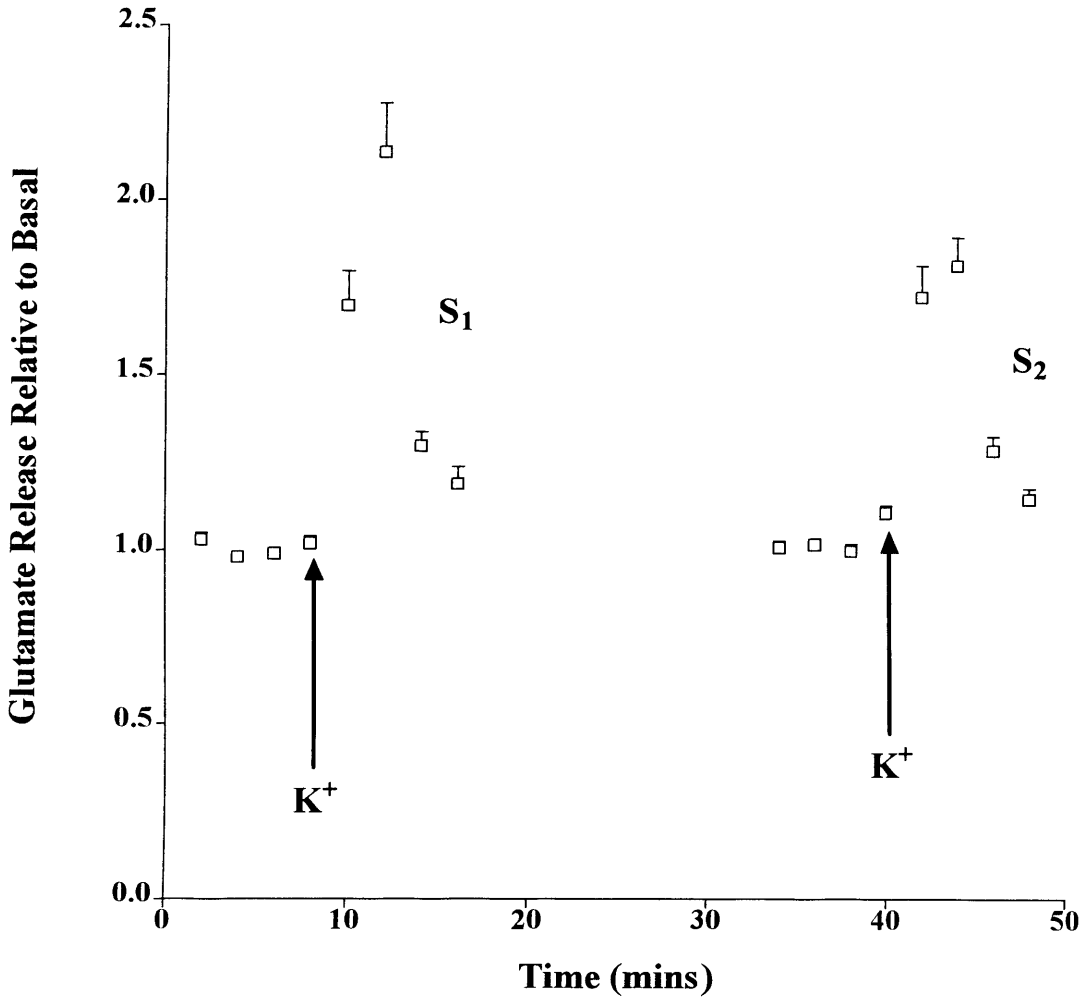


Fig 4.11. The effect of the application of 2×2min pulses of 46mM K^+ (S_1 and S_2) on glutamate release from rat cerebrocortical slices. Data are mean±sem (n=75).

Chapter 5. Effects of Opioids on 46mM K^+-evoked Glutamate Release from Rat Cerebrocortical Slices

5.1. Introduction

Opium (the main active constituent is morphine) has been used for many centuries for the relief of pain [Sertuner et al., 1806]. The term opioid refers to any substance that produces morphine-like effects, which can in turn be antagonised by naloxone. Opioid receptors belong to the family of G-protein coupled receptors. Their presumed mechanism of action is via a reduction in neurotransmission [Mulder 1993] primarily by closing voltage sensitive Ca^{2+} channels, hyperpolarising the cell membrane via an increased outward K^+ conductance and reducing cAMP and the inwardly rectifying K^+ current (I_h) [North 1993; Ingram and Williams 1994]. Opioids are the main therapeutic agents used in the pre-, intra- and postoperative periods for analgesia [Pasternak 1993]. In this area, morphine remains the most widely used analgesic for moderate to severe pain and is the standard with which all opioids are compared.

Opioid receptors are classified as μ, δ and κ and all have been cloned and sequenced (see Chapter 1, Section 1.83, page 25). A recent addition of the opioid receptor family was cloned in 1994, it did not belong to the classical opioid receptors (μ, δ or κ), and was termed the orphan receptor [Mollereau et al., 1994; Bunzow et al., 1994]. The identification of an endogenous ligand in 1995 for the orphan receptor termed nociceptin by Meunier et al., 1995 (extracted from whole rat brain), and orphanin FQ (O-FQ) by Reinsheid et al., 1995 (extracted from porcine hypothalamus) completed the story to date.

Aims

In the previous chapter a superfusion method was established, described and validated for the measurement of glutamate release from rat cerebrocortical slices. Using this method, the aims of this chapter are to characterise the effects of a range of opioid receptor sub-

type selective agonists and an opioid-like peptide (nociceptin), on the release process. These results may provide evidence for:

1. A role for opioids in the control of glutamate release.
2. The possible role for opioids as neuroprotectants, as suggested by Hayward et al., 1996.
3. A role for an opioid-like peptide (nociceptin) in the control of glutamate release.

5.2. Methods

Release Protocol

Cerebrocortical slices were prepared from female Wistar rats as previously described in Chapter 2, Section 2.4, page 43. The slices were agitated and washed, then 1ml was pipetted into a perfusion chamber as previously described in Chapter 4, page 83. Also in Chapter 4, page 84, are the details of perfusion rates, amount and times of fractions collected and the stimulation parameters for the slices. Briefly, the slices were perfused with bicarbonate buffer at a rate of 1ml/min for 60min, prior to collection of fractions. Fractions were collected every 2min for 6min before the addition of a 2min pulse of 46mM K^+ (S_1). 30min after S_1 a second 2min pulse of 46mM K^+ was applied (S_2). Four final fractions were collected after S_2. Analysis of the fractions collected for glutamate release were performed fluorimetrically as described in Chapter 4, page 84.

Addition of Agents

EGTA (a Ca^{2+}-chelating agent {0.1mM in Ca^{2+} free bicarbonate buffer}), morphine (0.01-10μM), DAMGO (1μM, μ-selective agonist), DPDPE (1 and 10μM, δ-selective agonist), ICI-199,441 (1 and 10μM, κ-selective agonist) and spiradoline (1 and 100μM, κ-selective agonist) were applied immediately after S_1 until the end of the experiment (also present during S_2). In some experiments involving DAMGO at 1μM and spiradoline at 100μM, the agents were added 10min after S_1 with the addition of cyprodime (10μM, μ-selective antagonist) and nor-binaltorphimine (nor-BNI) (10μM, κ-selective antagonist) immediately after S_1 respectively. The control slices for these experiments received the respective agonist at the same time. To examine the effects of DAMGO (1μM), DPDPE (1μM) and spiradoline (1 and 30μM) on basal glutamate release, these agents were added in place of S_2-46mM K^+ stimulation, until the end of the experiment. The purpose of including S_1 was as an internal control for tissue integrity.

Nociceptin (10-1000nM), in the presence of 30μM bestatin, amastatin, captopril, phosphoramidon and 0.1% Bovine Serum Albumin (BSA) to prevent breakdown was applied immediately after S_1 until the end of the experiment. In some experiments involving nociceptin at 100nM, naloxone (10μM) was added alone immediately after S_1

(until the end of the experiment), with the introduction of nociceptin 10min after naloxone. Control slices received nociceptin at the same time intervals.

Data and Statistical Analysis

In time course studies, glutamate release was expressed relative to the mean of the first three basal samples (=1.00). The S_2/S_1 ratios and the % inhibitions were also calculated, as described in detail in the formular in Chapter 4, page 85.

Data are presented as mean±sem (n). Statistical analysis was performed using Kruskal Wallis and Wilcoxon rank sum tests where appropriate, and considered significant when $p<0.05$.

5.3. Results

Effect of 46mM K^+-depolarisation

Depolarisation of rat cerebrocortical slices with 46mM K^+ produced a monophasic release of glutamate for both S_1 and S_2, (Fig 5.1). Under control conditions basal and evoked release amounted to 822.32±71.44 and 1552±163.6nmoles/2min samples (n=75). As there was considerable variation in the levels of stimulation between rats, the S_2/S_1 ratio was used to compare between stimulations of the same batch of slices. This enables the overall stimulation and not just the peak responses to be quantified. From 75 control experiments presented in this chapter, the mean S_2/S_1 ratio was 1.07±0.08, (range 0.48-3.15).

Ca^{2+}-dependency

Fig 5.2 illustrates that glutamate release is a Ca^{2+} regulated release process. Removal of Ca^{2+} from, and introduction of EGTA (0.1mM) to the bicarbonate buffer, reduced the S_2/S_1 ratio by 79.2±10.6% from 1.35±0.48 to 0.25±0.12.

Effect of μ-Opioid Selective Agents

Morphine produced a dose dependent inhibition of glutamate release with an estimated IC_{50} of 71nM (Fig 5.3). When comparing S_2/S_1 ratios with paired control samples, a maximum inhibition of 78±12.5% was achieved at 1μM.

At 1μM, the μ-selective agonist-DAMGO produced a 59.3±13.2% inhibition of glutamate release. This inhibition was reversed upon the addition of 10μM cyprodime (a μ-selective antagonist) (Fig 5.4).

Effect of δ-Opioid Selective Agent

1 and 10μM DPDPE failed to significantly alter the 46mM K^+-evoked glutamate release from rat cerebrocortical slices (Fig 5.5).

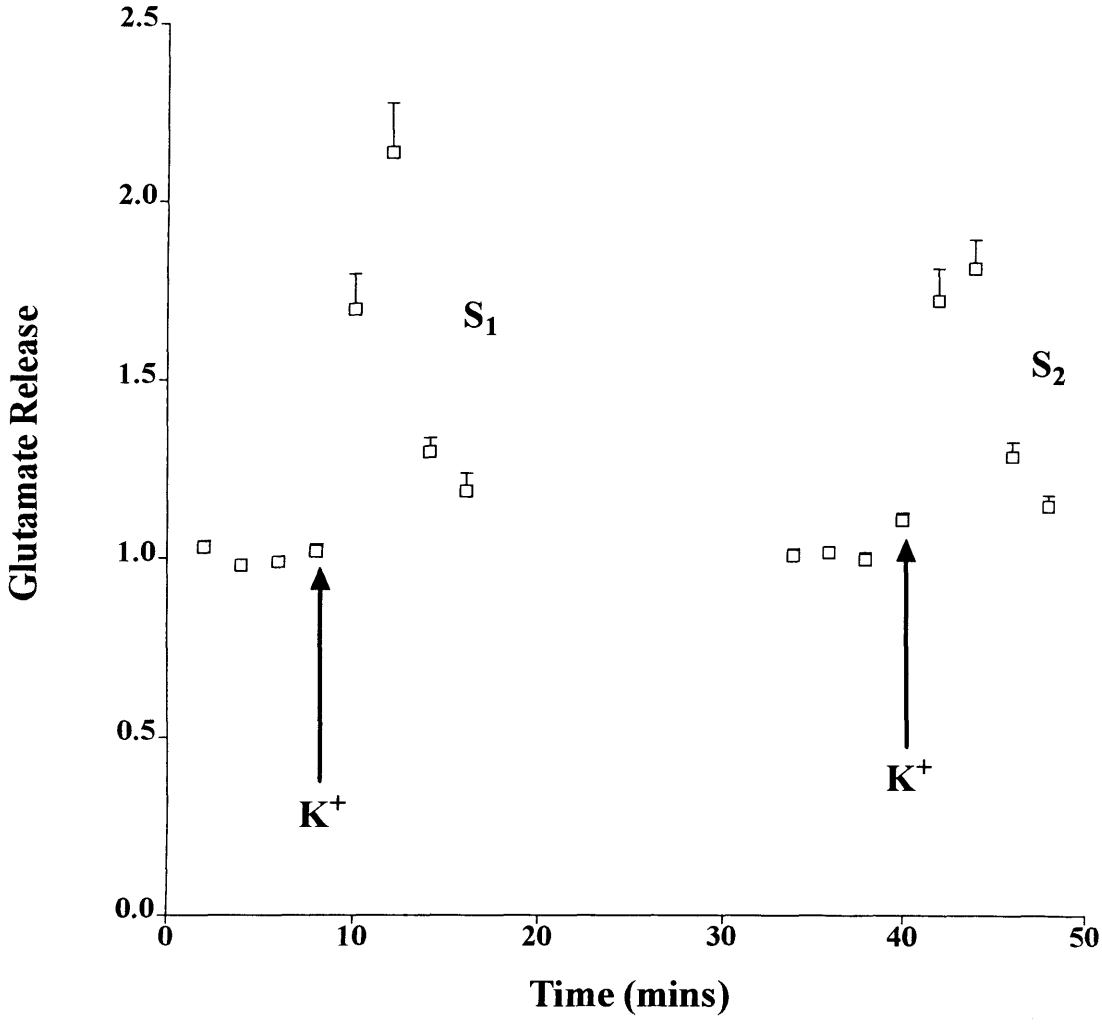


Fig 5.1. 2×2min pulses of 46mM K^+ (indicated by the arrows) produced monophasic releases of glutamate (S_1 and S_2) from perfused rat cerebrocortical slices. Data are mean±sem (n=75).

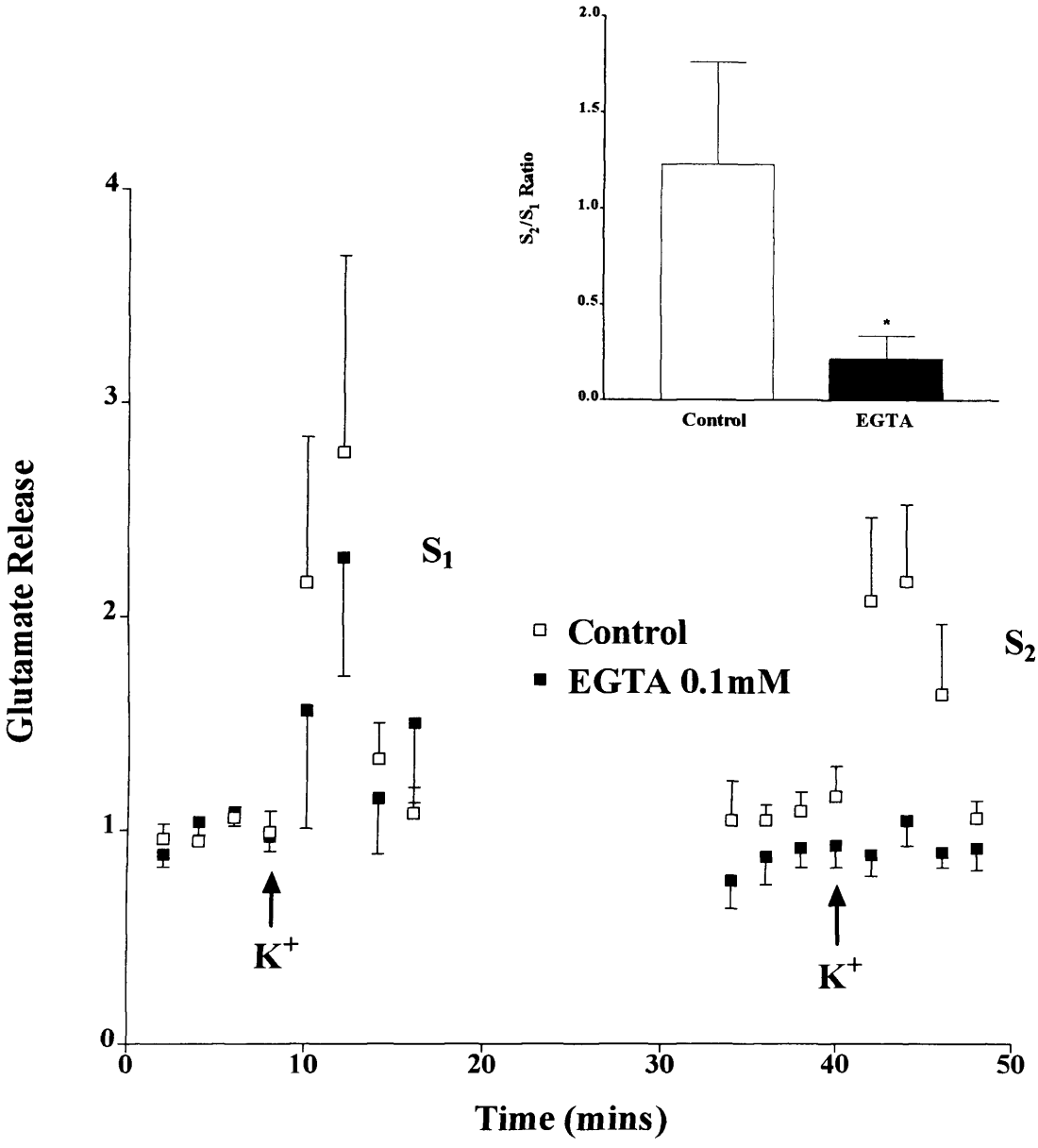


Fig 5.2. 0.1mM EGTA inhibits 46mM K^+-evoked glutamate release by 79.2±10.6%. The main picture illustrates the effect of EGTA on the time course of events, the inset illustrates the decrease in S_2/S_1 ratio compared with paired control. Data are mean±sem (n=5), and * illustrates statistical significance compared to paired control.

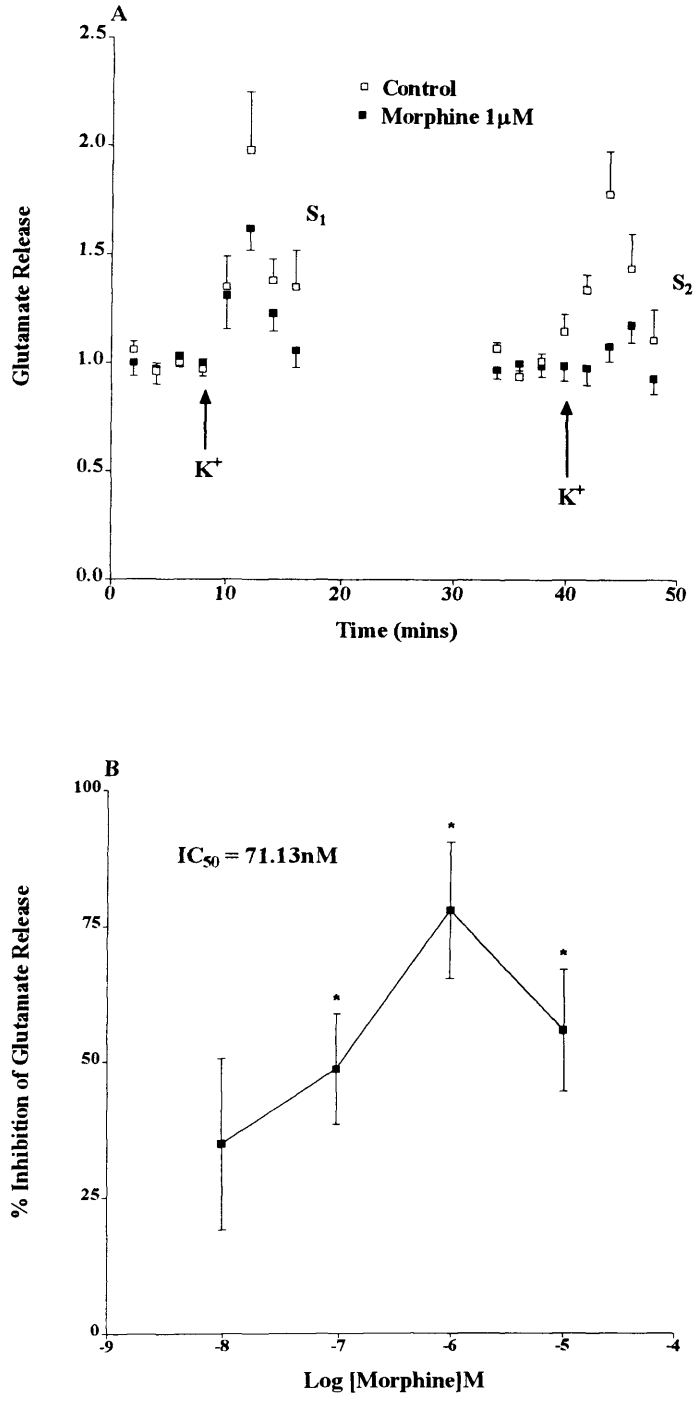


Fig 5.3. Morphine at 1μM inhibited 46mM K^+-evoked glutamate release by 78±12.5% (A). Morphine dose-dependently inhibited the S_2/S_1 ratio of 46mM K^+-evoked glutamate release from rat cerebrocortical slices compared to control with an estimated IC_{50} of 71nM(B). Data are mean±sem (n=6-13). * illustrates statistical significance compared with paired control, $p<0.05$ by Kruskal Wallis.

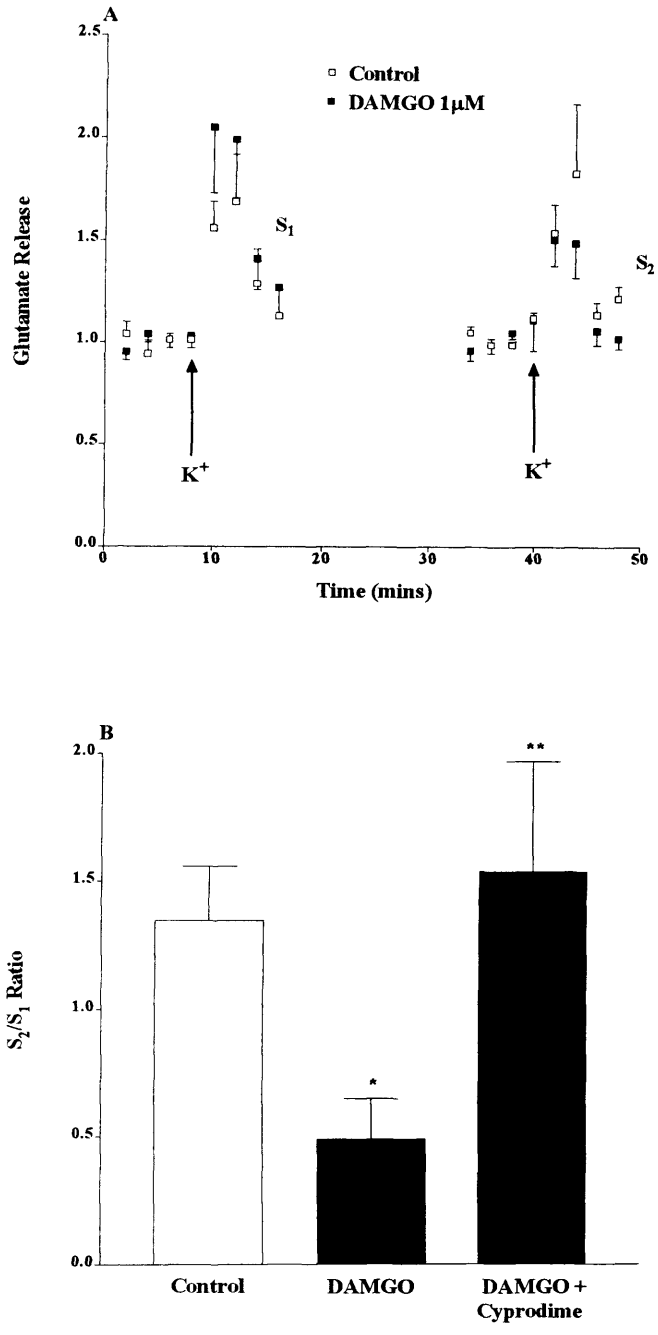


Fig 5.4. 1μM DAMGO inhibits 46mM K^+-evoked glutamate release by approximately 59.3±13.2% (A). The inhibition of S_2/S_1 ratio achieved by 1μM DAMGO, was reversed by 10μM cyprodime (B). Data are mean±sem (n=6), and * and ** illustrate statistical significance between control and DAMGO, and DAMGO+cyprodime and DAMGO alone, respectively.

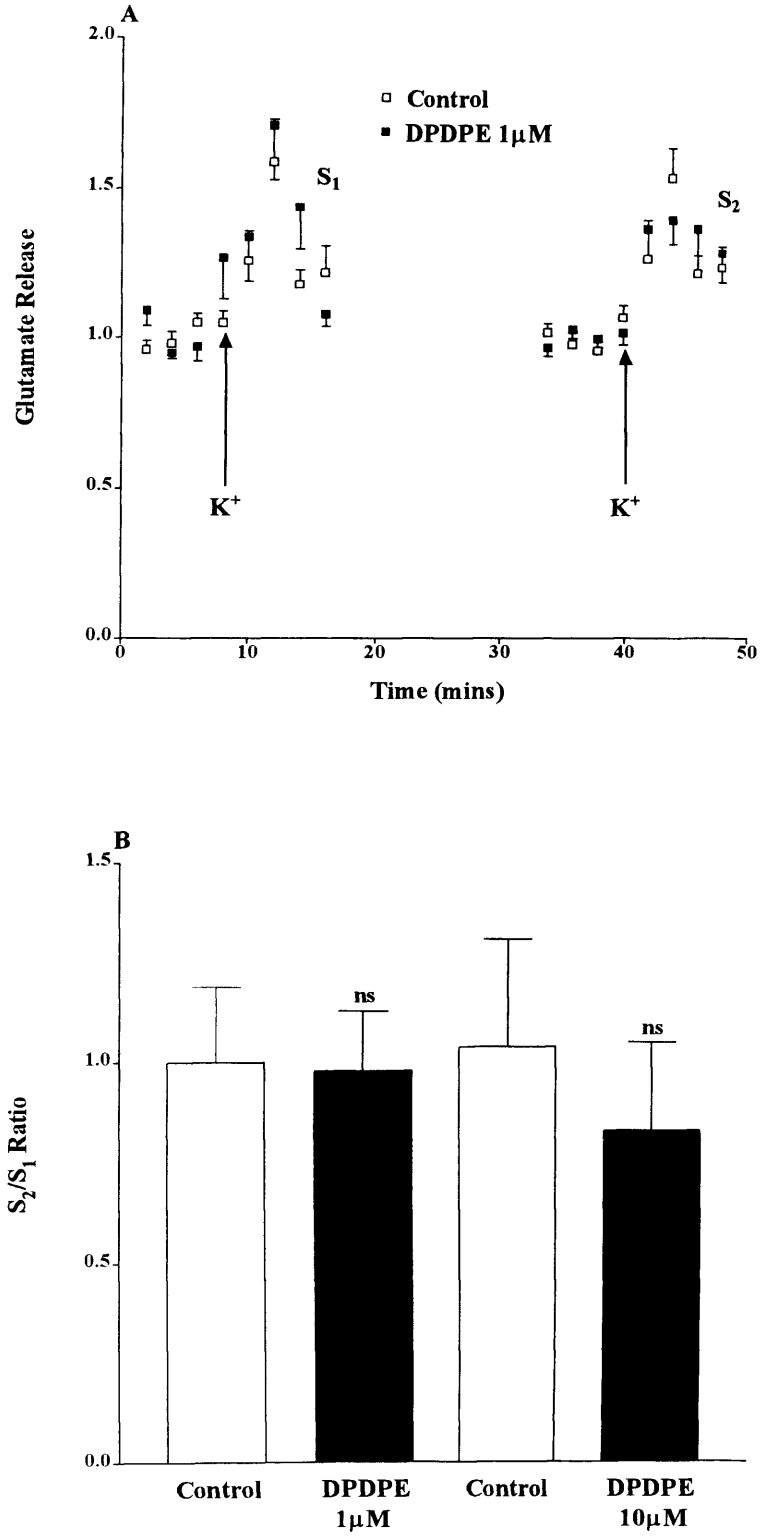


Fig 5.5. 1μM DPDPE did not alter 46mM K^+-evoked glutamate glutamate (A). Neither 1 or 10μM DPDPE altered S_2/S_1 ratio (B). Data are mean±sem (n=3-8) and ns=no statistical significance compared with control.

Effect of κ-Opioid Selective Agents

ICI-199,441 at 1μM did not significantly alter 46mM K^+-evoked glutamate release. At 10μM, there was a significant inhibition of 57.2±15%, reducing the S_2/S_1 ratio from 0.96±0.18 to 0.35±0.11 (Fig 5.6).

Spiradoline (1 and 100μM) produced a 52.8±13.7 and 71.3±10.5% inhibition, reducing the S_2/S_1 ratios from 1.29±0.26 to 0.50±0.16, and 1.04±0.15 to 0.24±0.08 respectively (Fig 5.7). The inhibitory effect of spiradoline at 100μM was reversed upon the addition of 10μM nor-binaltorphimine (a κ-selective antagonist) (Fig 5.8).

Effect of μ, δ and κ-Opioid Agonists on Basal Release

DAMGO (1μM), DPDPE (1μM) and spiradoline (1 and 30μM) did not alter basal glutamate release (Fig 5.9).

Effect of Nociceptin

Nociceptin produced a dose-dependent inhibition of 46mM K^+-evoked glutamate release, with a maximum inhibition of 71.7±7.7% achieved at 300nM (Fig 5.10). Further analysis of the dose response curve yielded an estimated IC_{50} value of approximately 51nM (Fig 5.10). 10μM Naloxone failed to reverse the inhibition produced by 100nM nociceptin (Fig 5.11).

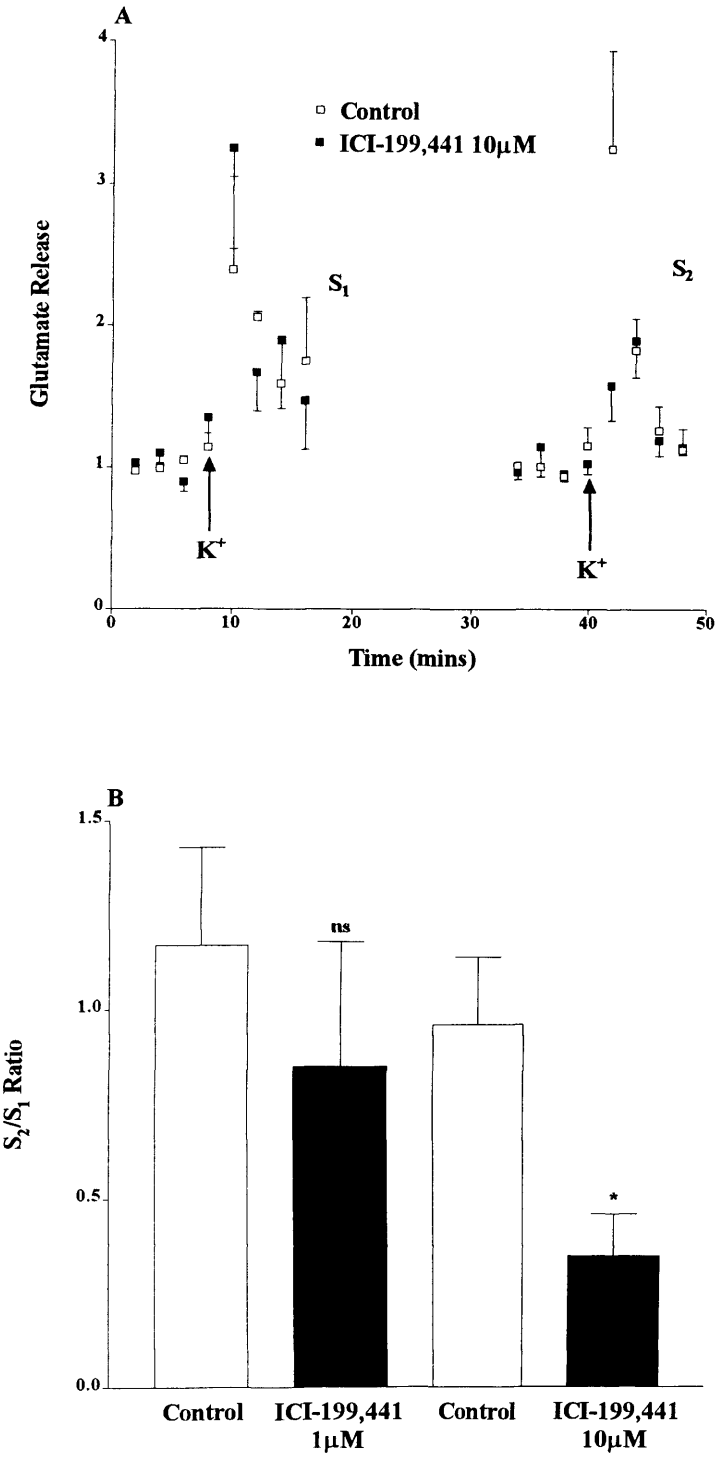


Fig 5.6. 10μM ICI-199,441 (A & B) inhibited 46mM K^+-evoked glutamate release by 57.2±15.1%. At 1μM ICI-199,441 no inhibition was achieved (B). Data are mean±sem (n=11). * illustrates statistical significance and ns=no statistical significance compared with paired control.

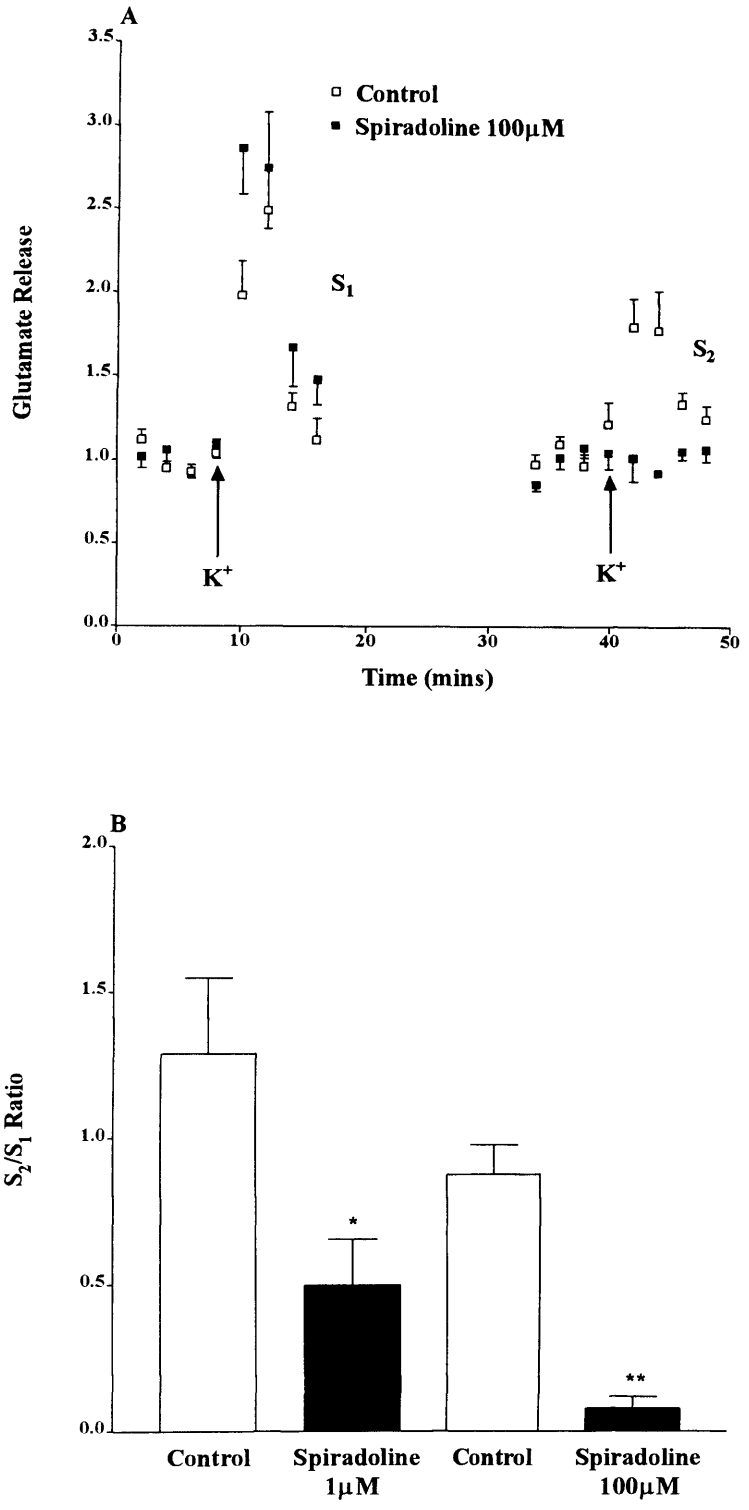


Fig 5.7. 100μM spiradoline (A & B) inhibited 46mM K^+-evoked glutamate release by 71.3±10.5%. 1μM spiradoline reduced the 46mM K^+-evoked glutamate release by 52.8±13.7% (B). Data are mean±sem (n=6) and * and ** illustrate statistical significance at 1 and 100μM respectively compared with paired control.

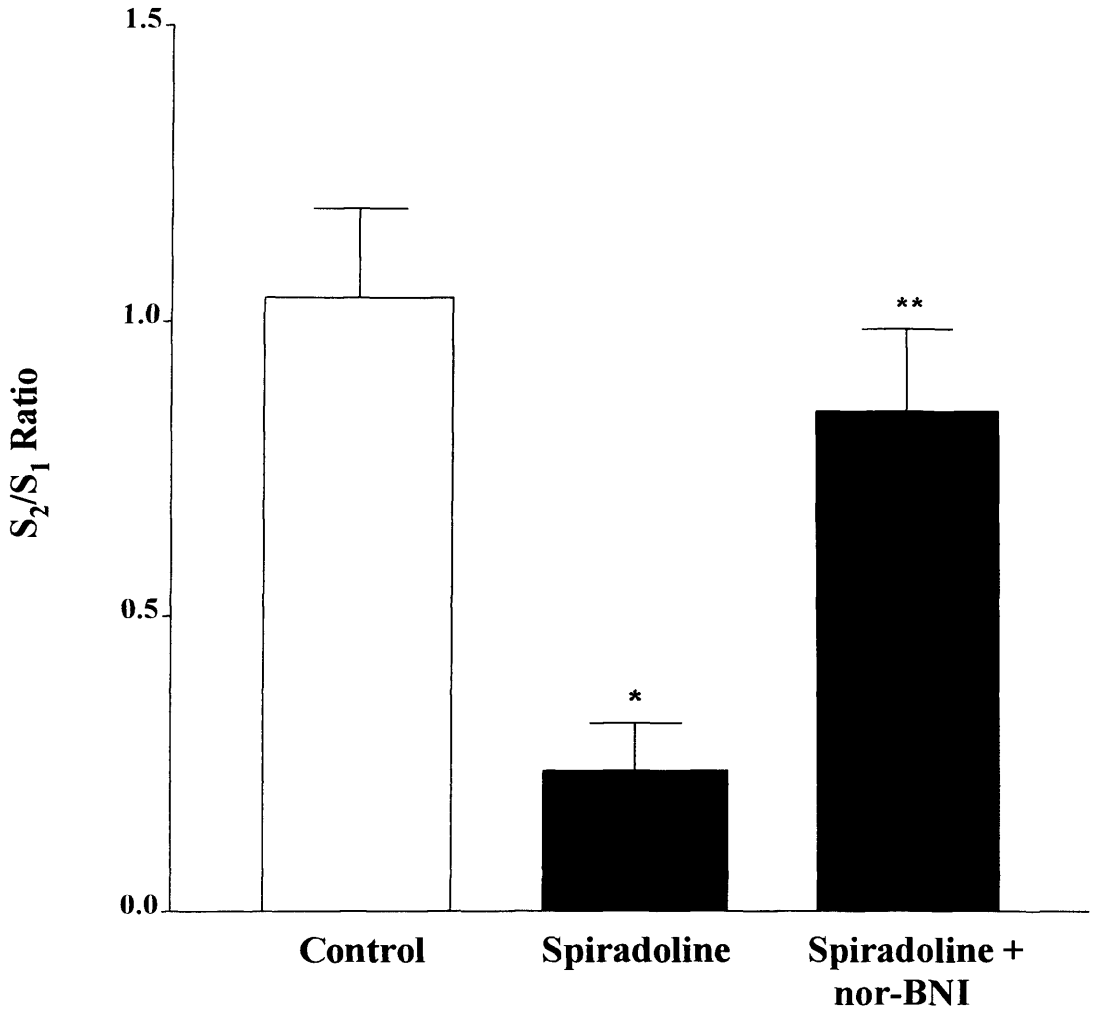


Fig 5.8. 100μM spiradoline inhibits 46mM K^+-evoked glutamate release by 71.3±10.5%. The addition of 10μM nor-BNI reversed the inhibition. Data are mean±sem (n=6) and * and ** illustrate statistical significance between control and spiradoline, and spiradoline+nor-BNI and spiradoline respectively.

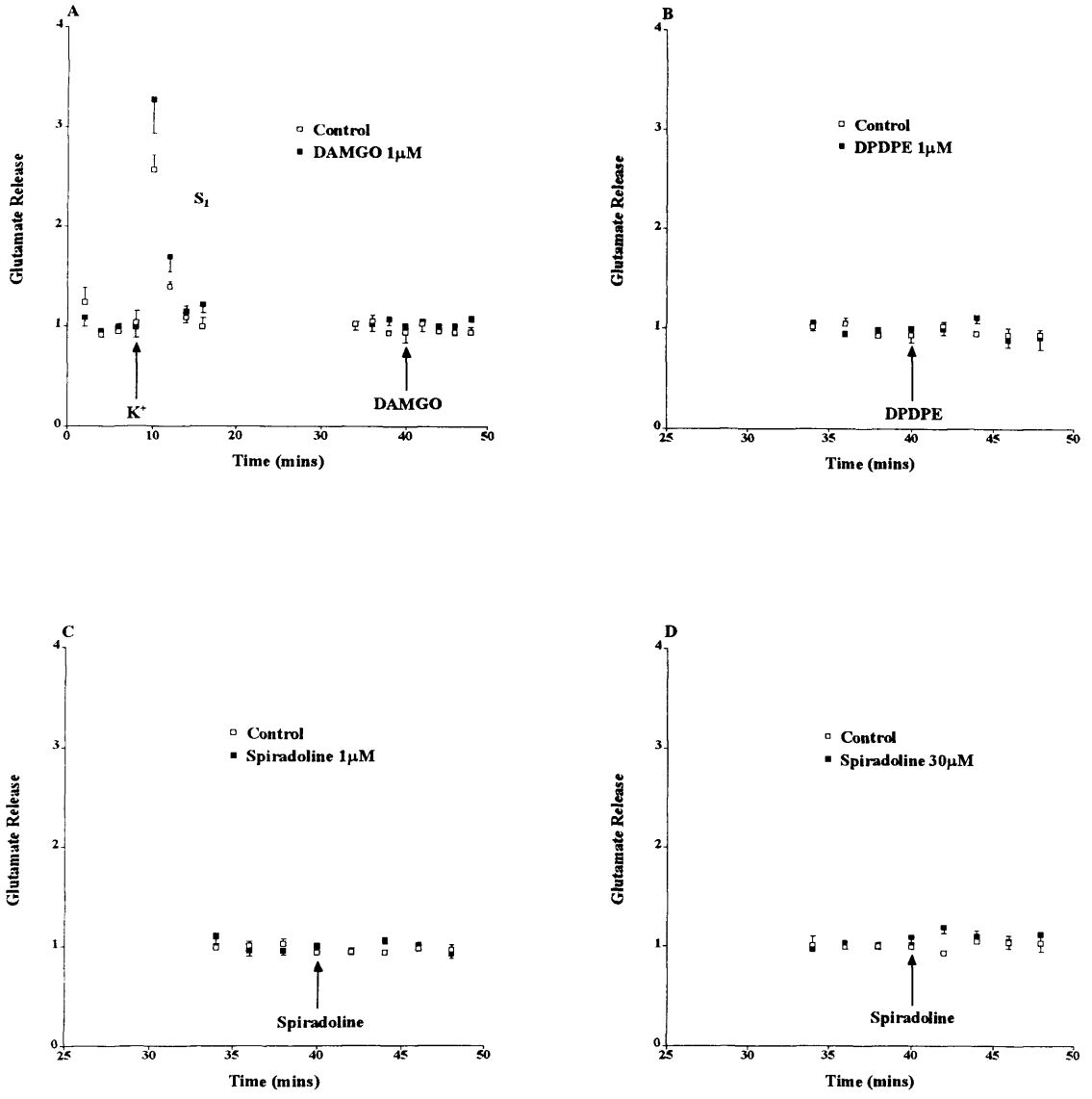


Fig 5.9. 1μM DAMGO (A), 1μM DPDPE (B), 1 (C) and 30μM (D) spiradoline fail to alter basal glutamate release from rat cerebrocortical slices. S_1 has been omitted from B, C and D for clarity of the results. Data are mean±sem (n=3).

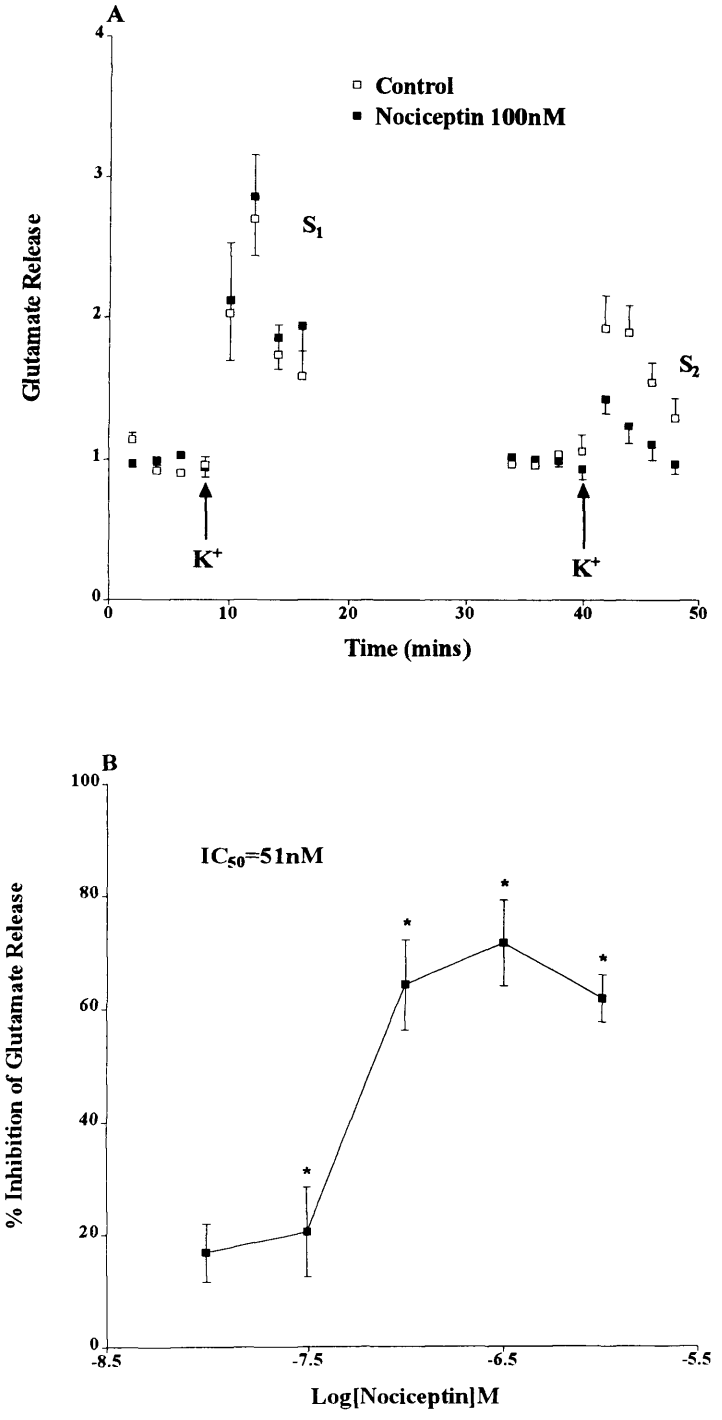


Fig 5.10. 100nM nociceptin inhibits 46mM K^+-evoked glutamate release by 64.3±8% (A). Nociceptin produced a dose-dependent inhibition of 46mM K^+-evoked glutamate release with an estimated IC_{50} of 51nM (B). Data are mean±sem (n=5-11). * illustrates statistical significance compared with paired control and $p<0.05$ by Kruskal Wallis.

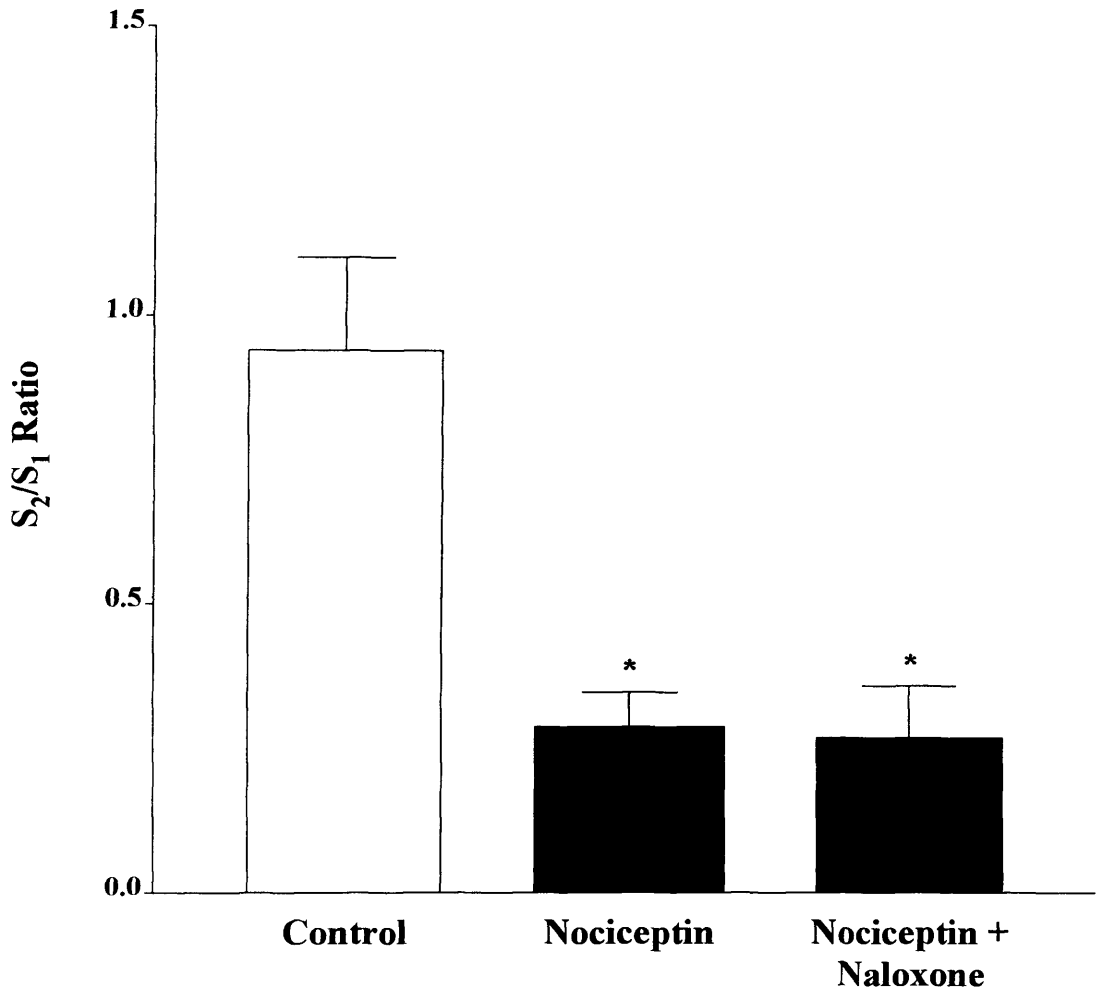


Fig 5.11. 100nM nociceptin inhibits 46mM K^+-evoked glutamate release by approximately 64.3±8%. The addition of 10μM naloxone failed to alter this inhibition. Data are mean±sem (n=5) and * illustrates statistical significance compared with control.

5.4. Conclusions

Depolarisation of perfused rat cerebrocortical slices with 46mM K^+ produced a monophasic release of glutamate for both S_1 and S_2. The release of glutamate was Ca^{2+}-sensitive, in that 0.1mM EGTA pre-treatment essentially abolished the evoked release confirming Ca^{2+} regulated exocytosis. Morphine produced a dose dependent inhibition of glutamate release. The inhibition of glutamate release achieved by DAMGO and spiradoline was reversed by cyprodime and nor-BNI respectively. DPDPE proved ineffective. Neither DAMGO, DPDPE nor spiradoline affected basal glutamate release. Nociceptin produced a dose dependent inhibition of glutamate release, irreversible by naloxone. The perfusion of slices and subsequent measurement of endogenous glutamate offers advantages over measurement of [^{3}H]L-glutamate overflow. Perfused tissue enables the dynamics of the release to be quantified, and the release will also eliminate the possibility of [^{3}H]L-glutamate-'pooling' (Chapter 4, Section 4.4, pages 81 & 82).

In the rat cerebrocortex, μ, δ and κ opioid receptors can be found in the frontal, piriform, and entorhinal regions in roughly equal proportions. However in the frontal cortex, μ receptor density is greater than that of κ, and δ lies somewhere in between [Mansour et al., 1988]. In this chapter, morphine and DAMGO (μ-agonists), 10μM ICI-199,441 and spiradoline (κ-agonists), have been shown to inhibit K^+-evoked release of glutamate.

The effects of opiates on the release of glutamate is controversial. In rat synaptosomes morphine did not affect K^+-evoked [^{3}H] glutamate release [Bartlett and Smith 1995]. The differences compared with the data presented here may be due to different tissue preparations. However, Bradford et al.; 1986, reported the inhibitory effects of μ and κ-opioid agonists on veratrine-stimulated release of glutamate. An inhibitory effect on capsaicin-evoked glutamate release from rat spinal dorsal horn slices was produced by both DAMGO and morphine (reversible by 1μM naloxone) but not by the κ opioid agonists U-50,488H and U-69,593 [Ueda et al., 1995]. At variance with the general ineffectiveness of κ-agonists reported by Ueda et al., 1995, enadoline (a κ-agonist) has been shown to inhibit glutamate release from ischaemic brain and consequently reduce cerebral damage in the cerebral cortex of rats [Hayward et al., 1995]; decrease 4-AP

stimulated release of glutamate from rodent and primate striatum (reversible upon the addition of 5μM nor-BNI) [Hill and Brotchie 1995]; reduce K^+-evoked release of glutamate from slices of substantia nigra in a concentration dependent manner, (also reversed by nor-BNI) [Maneuf et al., 1995]. In addition, endogenous dynorphins (κ-agonists) have also been reported to block the induction of hippocampal long term potentiation [Wagner et al., 1993]. The inhibitory effects seen with κ-agonists in this chapter were achieved at high concentrations, which may account for this anomaly. However, the inhibition produced by DAMGO and spiradoline in this chapter is likely to be due to μ and κ receptor activation, as the selective antagonists cyprodime and nor-BNI reversed the observed inhibition.

The failure of DPDPE to inhibit glutamate release is more difficult to explain. There are reports showing that 100μM DPDPE produced an inhibition of veratrine-stimulated glutamate release [Bradford et al., 1986], and 10μM DPDPE reduced the capsaicin-evoked glutamate release, in a naltrindole sensitive fashion [Ueda et al., 1995]. In this chapter a concentration of 1μM DPDPE was used, a lower concentration than either of the above studies. Yet when a limited number of experiments (n=3) were carried out using a higher concentration of DPDPE (10μM), no statistically significant inhibition was observed. Furthermore, in a series of experiments performed in our laboratory examining the effects of DPDPE on cAMP and IP_3 formation in Chinese hamster ovary cells expressing recombinant δ receptors, 1μM DPDPE produced a supra maximal response [Hirst et al., 1996; Smart et al., 1996].

Nociceptin was shown to inhibit 46mM K^+-evoked glutamate release from rat cerebrocortical slices, in a concentration-dependent manner with an estimated IC_{50} of 51nM. This represents the first report of ORL_1 coupling to glutamate release. The inhibition achieved with nociceptin was in the presence of the peptidase inhibitors: amastatin, bestatin, captopril and phosphoramidon and bovine serum albumin (BSA). These agents inhibit the peptidase enzymes that would breakdown nociceptin during perfusion of the cerebrocortical slices and BSA reduces the adsorption of the peptide into the perfusion circuit. ORL_1 receptor transcripts can be found in high levels in limbic regions such as the cerebral cortex, amygdala and hippocampus [Bunzow et al., 1994;

Mollereau et al., 1994; Julius 1995; Lachowicz et al., 1995]. Utilising radioligand binding studies, [^{3}H]-nociceptin has been reported to label a number of binding sites within the guinea-pig CNS [Paterson and McKnight 1996], and in membranes from chinese hamster overy (CHO) cells expressing ORL_1 receptor [Butour et al., 1997]. In homogentes of guinea-pig brain, [^{3}H]-nociceptin labels a single population of binding sites with a K_d of 0.134±0.03nM and a binding capacity of 9.78±1.9pmol/g. The sites labelled with nociceptin were unlikely to be that of a sub-type of the opioid-receptor as the binding was not displaced by classical opioid ligands, and the binding capacity was greater than that of any of the μ-, δ- or κ-opioid sites [Paterson and McKnight 1996]. Butour et al., 1997 reported that the binding of [^{3}H]-nocieptin to membranes of CHO cells expressing the ORL_1 receptor was saturable (Bmax 1.3-1.8pmol/mg protein) and of high affinity (K_d ~0.1nM), although B_{max} will clearly depend on the transfection procedure.

The estimated IC_{50} value of 51nM for the inhibition by nociceptin of 46mM K^+-evoked glutamate release found in this chapter, is comparable with IC/EC_{50} values for a number of cellular responses. IC/EC_{50} values of 42 and 60nM have been reported by Connor et al., 1996B, in SH-SY5Y human neuroblastoma cells for N-type Ca^{2+} channel current inhibition and the enhancement of carbachol-stimulated increase in $[Ca^{2+}]_i$ respectively. Nociceptin has been shown to dose-dependently enhance an outward K^+ conductance in dorsal raphe neurons, with an estimated EC_{50} of 12nM [Vaughan & Christie 1996]. Increases in the inwardly rectifying K^+ conductance in rat locus coeruleus (LC) neurons have been reported upon the addition of nociceptin, by Connor et al., 1996B, where the results were consistent with the reported presence of mRNA for ORL_1 [Fukuda et al., 1994; Bunzow et al., 1994]. The glutamate inhibition observed in this study, irreversible by the addition of naloxone, may be due to a combination of Ca^{2+} channel inhibition and an enhancement of outward K^+ fluxes, resulting in hyperpolarisation. Nociceptin also decreases acetylcholine release from parasympathetic nerves innervating the guinea-pig trachea in a naloxone insensitive fashion [Patel et al., 1997].

Controversy surrounds the role for nociceptin/O-FQ at the ORL_1 receptor. Intra-thecal O-FQ was reported to induce dose-dependent antinociception in rats [Xu et al., 1996]. O-FQ was also reported by Mogil et al., 1996 to produce a functional antagonism of μ-, δ- and κ-

opioid antinociception following intracerebraventricular injections in mice. However, Stanfa et al., 1996, reported an inhibitory action of 225μg nociceptin on the C-fibre evoked wind-up and post-discharge of spinal dorsal horn neurons in the rat. This inhibitory effect was irreversible upon the addition of 10μg naloxone, but reversible when the dose was increased to 50μg. This is the first study to demonstrate a potential inhibitory action of nociceptin on wind-up and post-discharge of spinal neurons (i.e. analgesia), and is at variance with the reports of hyperalgesia following supraspinal administration [Meunier et al., 1995; Reinscheid et al., 1995]. However the results are in keeping with in vitro studies demonstrating nociceptin enhancement of K^+ conductance [Vaughan & Christie 1996] and inhibition of VSCCs [Connor et al., 1996B]. In contrast, Mogil et al., 1996, reported that intracerebroventricular injections of orphanin FQ in mice did not produce hyperalgesia, but reversed opioid mediated antinociception and also dose-dependently reversed systemic morphine antinociception. The ineffectiveness of naloxone to reverse the inhibitory effect of nociceptin in this chapter, suggests that the action of nociceptin may be at non-μ-, δ- and κ-opioid receptors and be specific for ORL_1 occupation.

ORL_1 receptor is also located in the ventral horn of the spinal cord, cortex, pontine nuclei and red nucleus, [Bunzow et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Mollereau et al., 1994; Wick et al., 1994], suggesting that nociceptin may play a role in the regulation of motor behavior. Devine et al., 1996 reported that microinjections of O-FQ failed to produce motivational effects even at doses that disrupted motor behaviour, indicating that this novel peptide did not possess abuse liability, in contrast with the potential for abuse that is inherent with morphine and classical opiates [Wise 1989]. The clinical potential of the ORL_1 receptor nociceptin system remains to be fully explored.

Chapter 6. Effects of Intravenous and Inhalation Anaesthetic Agents on 46m K^+-Evoked Glutamate Release from Rat Cerebrocortical Slices

6.1. Introduction

The molecular basis of the action of general anaesthetic agents remains unclear. In 1952 Larrabee and Posternak, examined the selective actions of anaesthetics on synapses and axons in mammalian sympathetic ganglia. They proposed that general anaesthetic agents have a more pronounced effect on synaptic mechanisms in the central nervous system (CNS), than on the propagation of electrical signals along axons. This is now a commonly held view [Griffiths and Norman 1993; Franks and Lieb 1994].

Do anaesthetic agents act generally to produce a range of metabolic modifications resulting in anaesthesia, or, as proposed by Franks and Lieb in 1984, act on specific neuronal sites? Anaesthetics inhibit the activity of the lipid -free enzyme luciferase [Franks and Lieb 1984], and produce stereoselective actions on ion channels [Franks and Lieb 1991], providing evidence that the action of anaesthetic agents may be at specific sites [Dildy-Mayfield et al., 1996]. Application of anaesthetics leading to an unconscious state, could be attributed to a potentiation of inhibitory post synaptic potentials [Richards 1983], or the depression of excitatory synapses [Puil and El-Beheiry 1990]. There is a good deal of evidence to support an interaction of a range of anaesthetic agents with the $GABA_A$ receptor (potentiating its inhibitory actions) and glutamatergic transmission (inhibiting its excitatory actions), see Chapter 1, Section 1.10.2.2, pages 36-38.

Aims

In Chapter 4 a method for the measurement of glutamate release was described and validated. This has been used to examine the effects of a range of anaesthetic agents on the release process for the following reasons:

1. Glutamate is the major excitatory neurotransmitter within the CNS, and any reduction in excitatory transmission may result in CNS depression and perhaps anaesthesia.

2. Anaesthetic agents like thiopentone are "neuroprotective" [Sano et al., 1993]. Propofol and barbiturates are agents commonly used to treat increases in intracranial pressure and provide sedation in head injury patients. The neuroprotective effect may result from a reduction in glutamate release.

6.2. Methods

Release Protocol

Cerebrocortical slices were prepared from female Wistar rats as previously described in Chapter 2, Section 2.4, page 43. The slices were washed and agitated for 40min, prior to pipetting approximately 1ml of gravity packed slices into a perfusion chamber as previously described in Chapter 4, Section 4.5, page 83. Also in Chapter 4, Section 4.5, page 84, are the perfusion rate, the amount and times of fractions collected and the stimulation parameters for the slices. Briefly, the slices were perfused with oxygenated bicarbonate buffer at a rate of 1ml/min for 60min, prior to collection of fractions. Fractions were collected every 2min for 6min before the addition of a 2min pulse of 46mM K^+ (S_1). 30min after S_1 a second 2min pulse of 46mM K^+ was applied (S_2). Four final fractions were collected after S_2. Analysis of the fractions collected for glutamate release were performed fluorimetrically as described in Chapter 4, Section 4.5, page 84.

Introduction of Agents

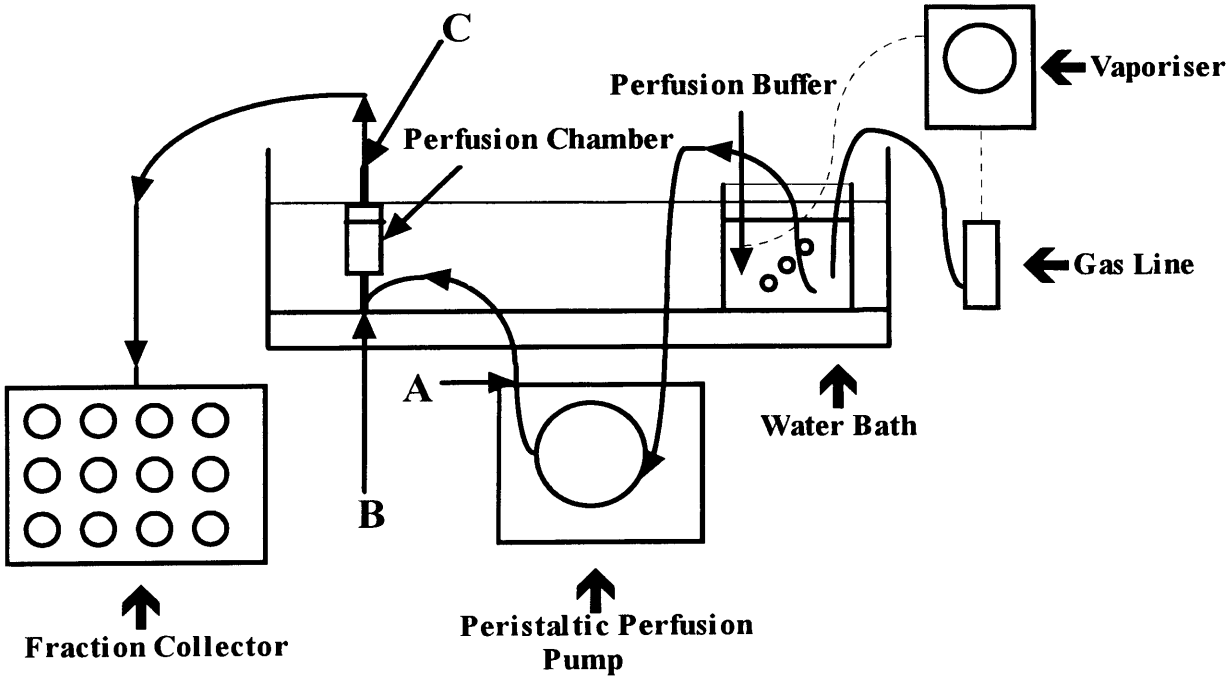
Ketamine, thiopentone and propofol (3, 10, 30, 100 and 300μM), MK-801 (1μM), pentobarbital (100μM), barbituric acid (300μM), halothane (at various vaporiser settings) and isoflurane (5% vaporiser setting) were introduced to the oxygenated bicarbonate buffer immediately after S_1 until the end of the experiment (thus including S_2).

Miscellaneous Methods

Prior to the examination of the effects of halothane and isoflurane on evoked glutamate release, the aqueous concentrations of the inhalation agents present in the bicarbonate buffer were measured by Gas Chromatography (GC) (Chapter 2, Section 2.10, page 46). A range of halothane partial pressures were delivered to the bicarbonate buffer for various time intervals using a precalibrated Fluotec 3 vaporiser and humidified carbogen (2.0 litre min^{-1} at 37°C), as the carrier gas. Aqueous halothane concentrations in the bicarbonate buffer were measured after 5, 10, 15, 20, 30, 45 and 60min by GC. The aqueous concentrations of halothane present in the tubing, exiting the peristaltic pump (A), just before entry to the perfusion chamber (B), and in the bicarbonate buffer exiting the perfusion chamber (C) were also measured by GC (Fig 6.1). The aqueous concentrations

of isoflurane (at a vaporiser setting of 5%), present in the bicarbonate buffer and exiting the perfusion chamber were also measured by GC (Fig 6.1).

Fig 6.1. Diagramatic representation of the perfusion apparatus. The vaporiser is connected to the gas line shown as a dotted line as it was not always connected. A, B and C represent the points within the perfusion system where samples were taken for measurement of the aqueous volatile concentration.



6.3. Results

Effect of Ketamine, MK-801 and Propofol

2x2min pulses of 46mM K^+ produced monophasic releases of glutamate from rat cerebrocortical slices, with a mean S_2/S_1 ratio of 1.06±0.05 (71). Ketamine produced a dose-dependent inhibition of 46mM K^+ evoked glutamate release from rat cerebrocortical slices (Fig 6.2 & 6.3) with a maximum inhibition of 65.1±8.1% ($p<0.05$) occurring at 300μM (Fig 6.2A). At 3μM no significant inhibition was achieved (Fig 6.2B). Further analysis of the dose response curve yielded an IC_{50} value of 18.2μM (Fig 6.3).

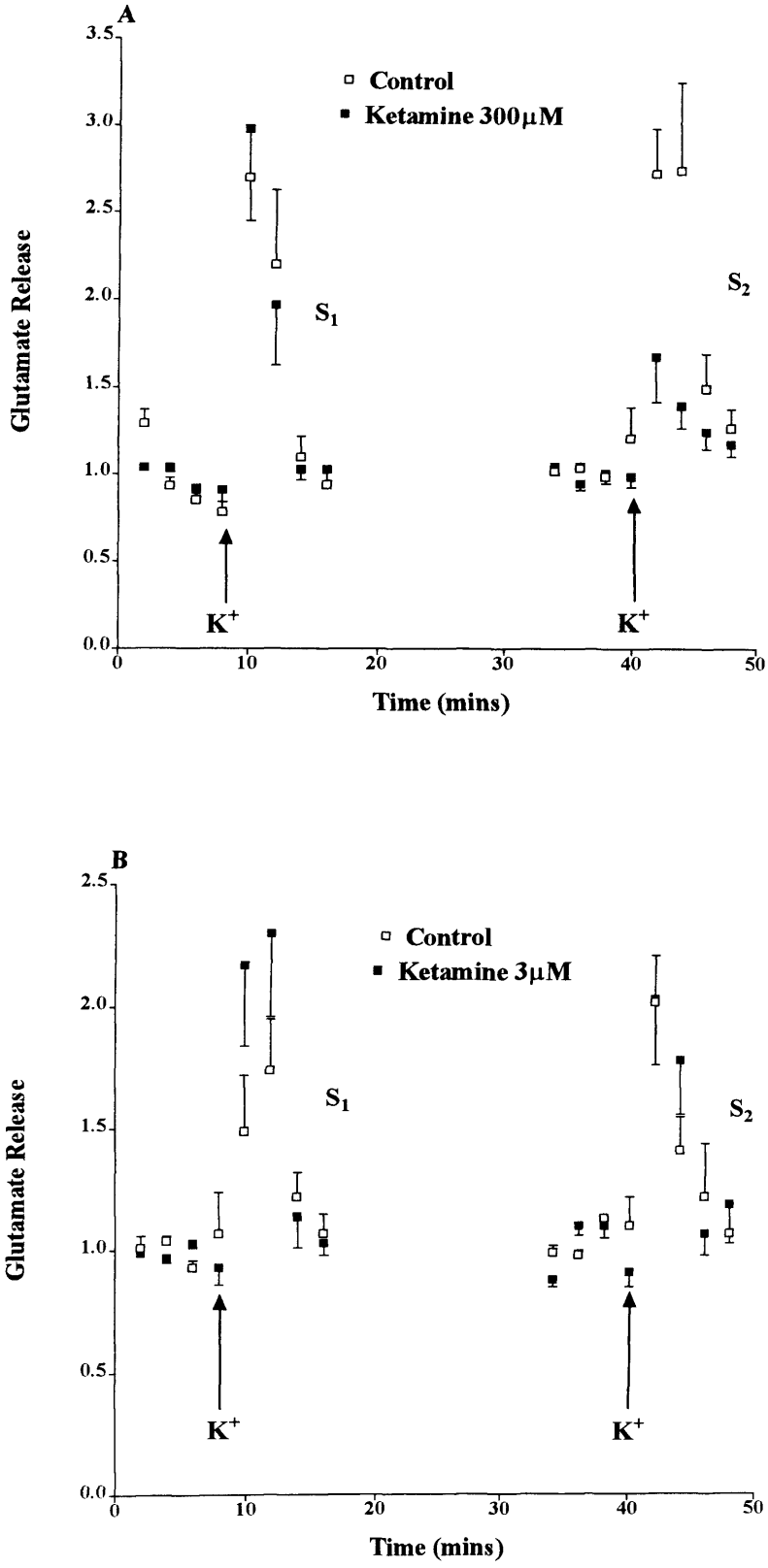
In addition, MK-801 at 1μM produced a 81.2±7.5% ($p<0.05$) inhibition compared with control slices (Fig 6.4).

Propofol produced a dose-dependent inhibition of 46mM K^+-evoked release of glutamate from rat cerebrocortical slices (Figs 6.5 & 6.6) with an estimated IC_{50} of 20.72μM. A maximum inhibition of 74.8±8.2% ($p<0.05$) was achieved at 300μM (Fig 6.5A), but no significant inhibition was achieved at 3μM (Fig 6.5B).

Effect of Barbiturates

Thiopentone produced a dose-dependent inhibition of 46mM K^+-evoked glutamate release from rat cerebrocortical slices (Fig 6.7 & 6.8) with a maximum inhibition of 55.4±16.8% ($p<0.05$) achieved at 300μM (Fig 6.7A). No statistical inhibition was achieved at 3μM (Fig 6.7B). Further analysis of the dose response curve yielded an IC_{50} value of 10.94μM (Fig 6.8).

Pentobarbital at 100μM, significantly reduced the 46mM K^+-evoked release of glutamate by 60.4±14.4% ($p<0.05$) (Fig 6.9A & 6.10). Barbituric acid at 300μM was ineffective (Fig 6.9B and Fig 6.10).



Figs 6.2. Ketamine at 300μM (A) inhibited 46mM K^+-evoked glutamate release by 65.14±8.13%. Ketamine at 3μM was ineffective. Data are mean±sem (n=5-7).

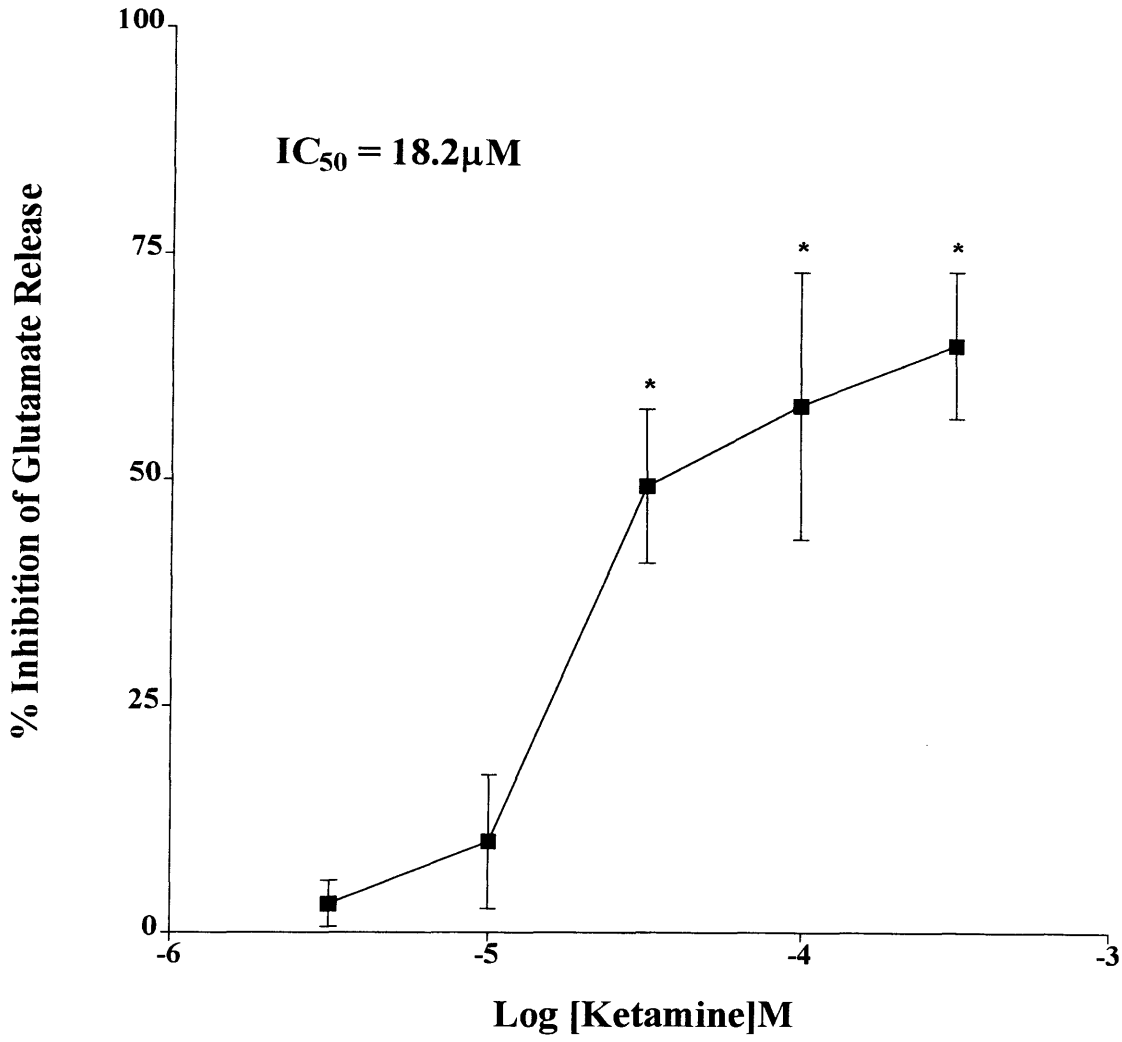


Fig 6.3. Ketamine produced a dose-dependent inhibition of 46mM K^+-evoked release of glutamate from rat cerebrocortical slices with an IC_{50} of 18.2μM. Data are mean±sem (n=5-9). * illustrates statistical significance compared with control and $p<0.05$ by Kruskal Wallis.

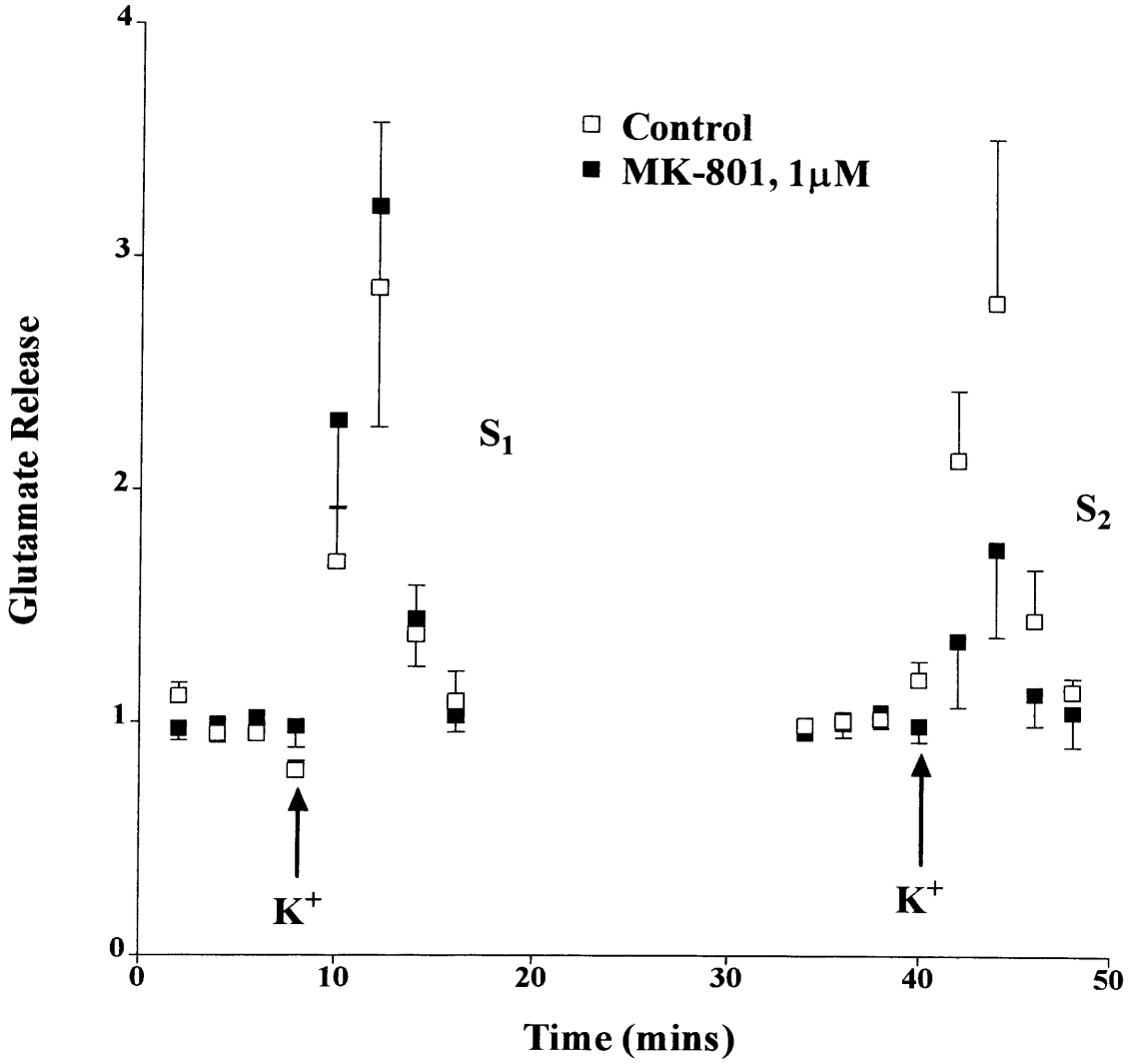
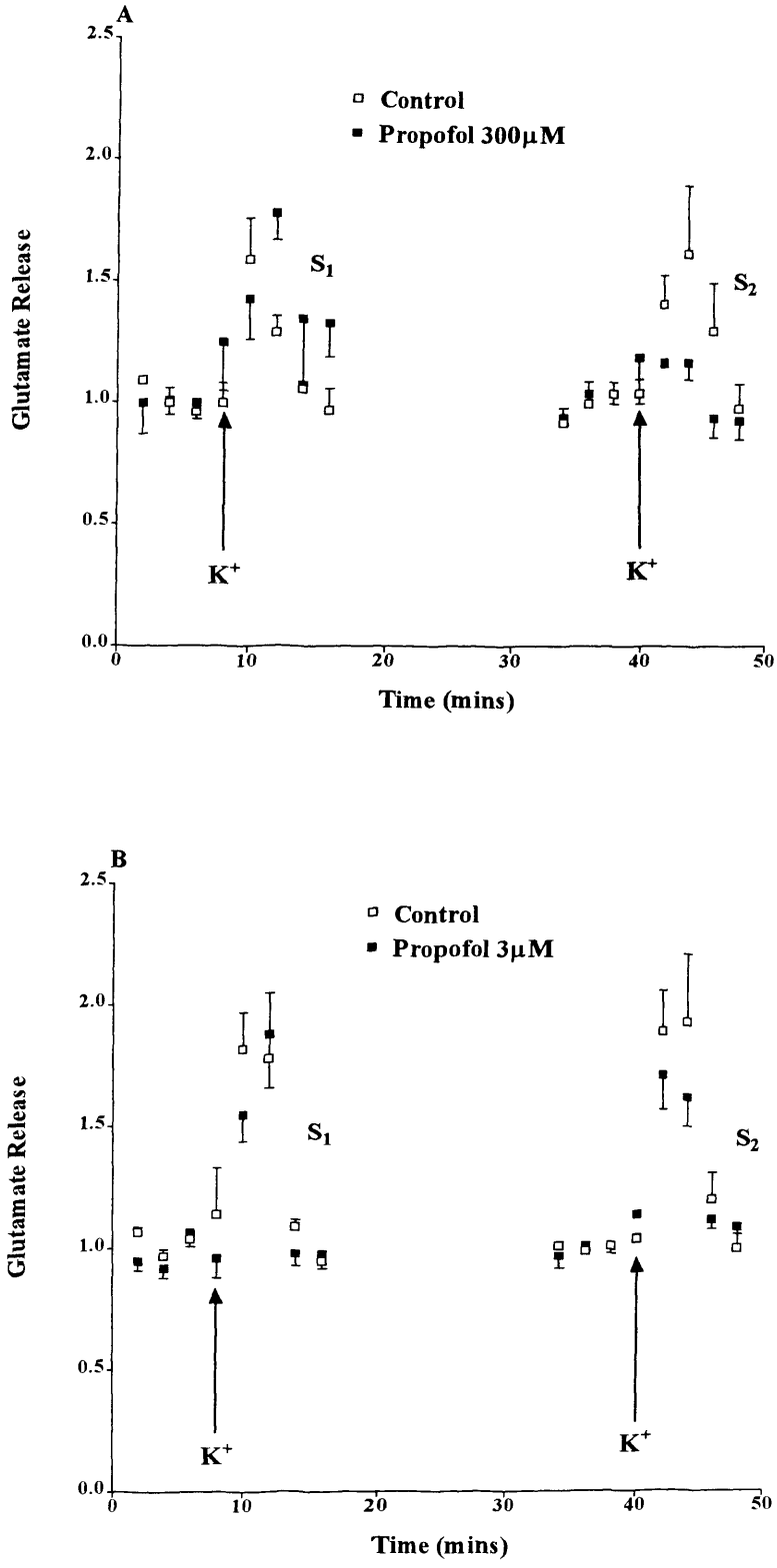


Fig 6.4. MK-801 at 1μM produced a 81.1±7.5% inhibition of 46mM K^+-evoked release of glutamate from rat cerebrocortical slices. Data are mean±sem, n=6.



Figs 6.5. Propofol at 300μM (A) inhibited 46mM K^+-evoked glutamate release by 74.8±8.2%. Propofol at 3μM (B) was ineffective. Data are mean±sem (n=5-7).

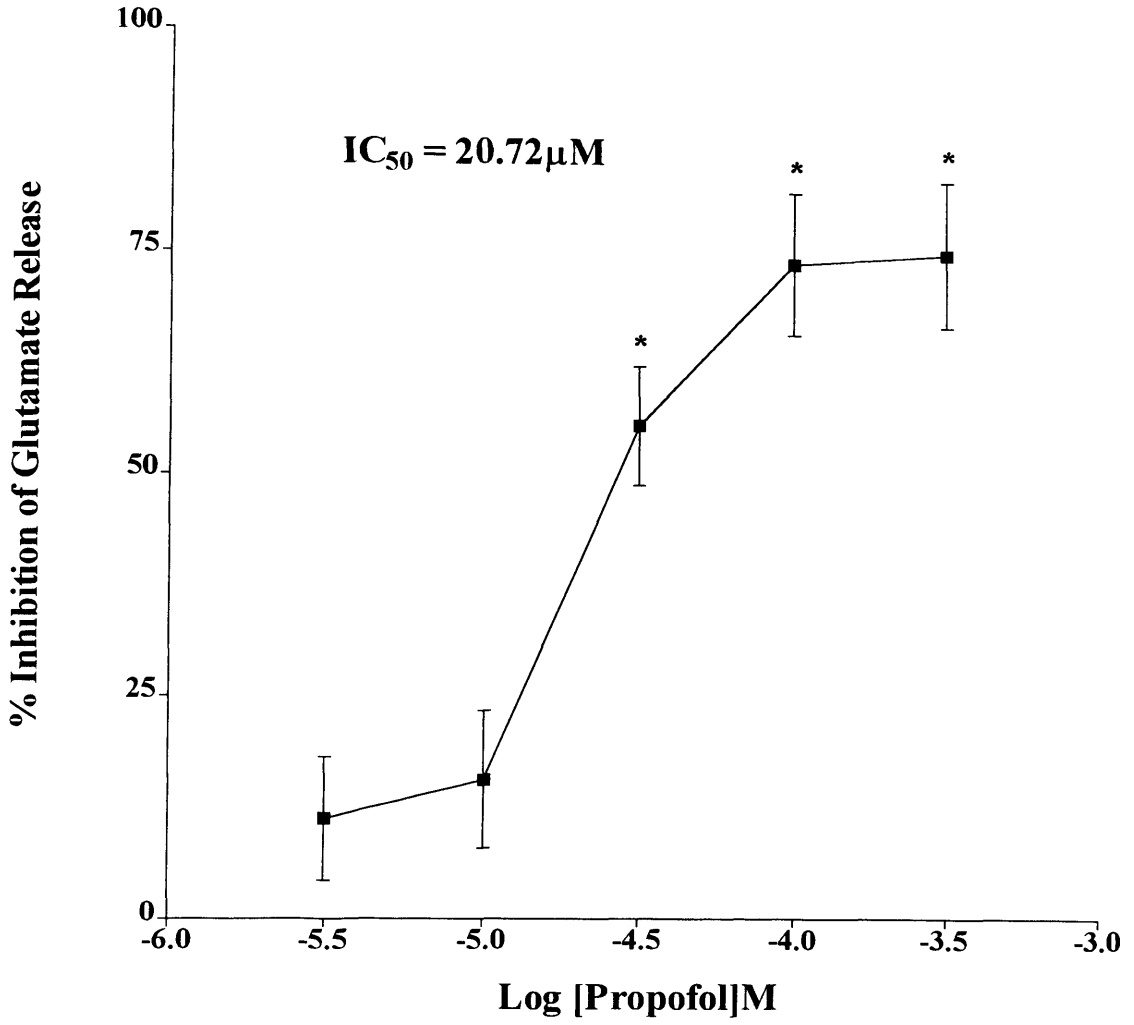
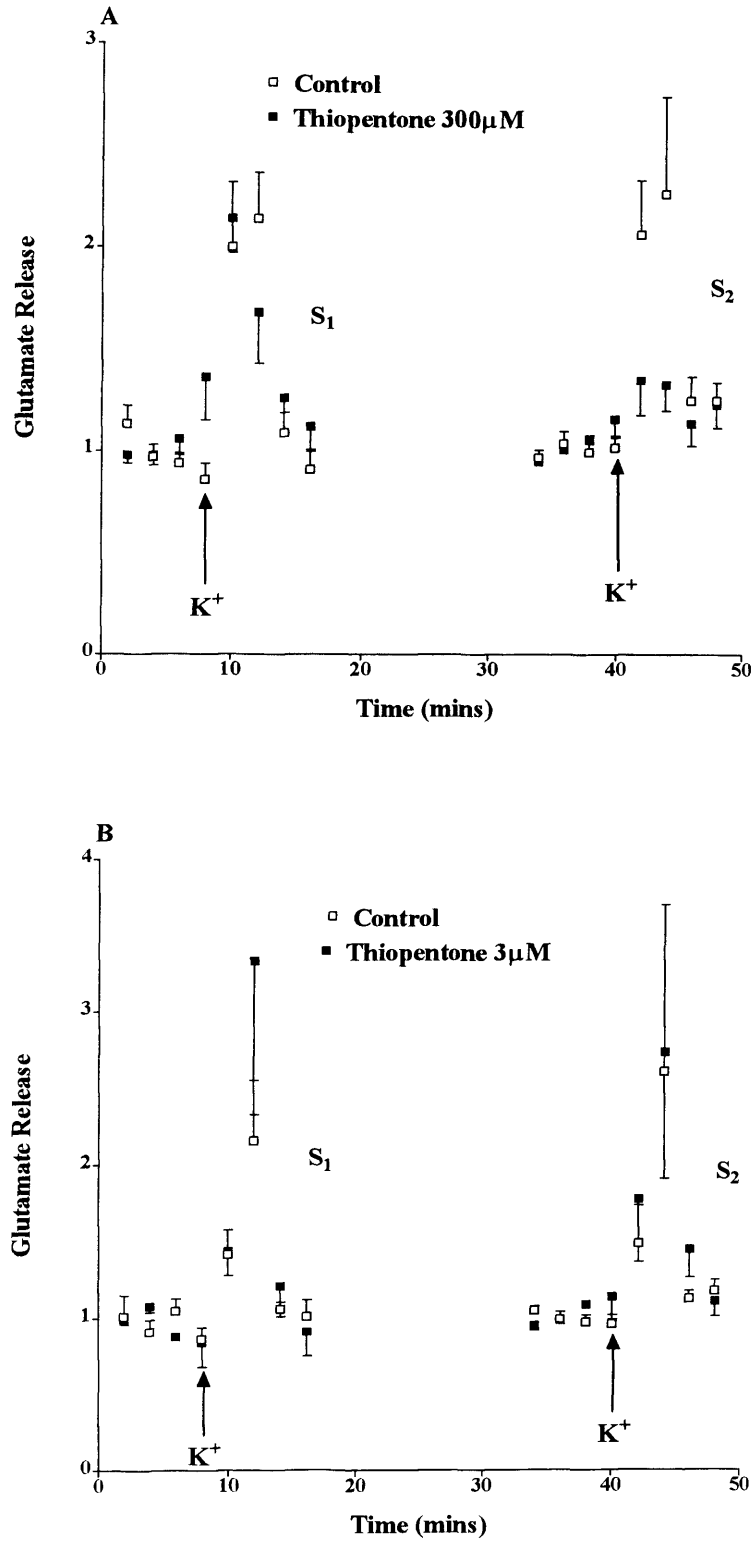


Fig 6.6. Propofol produced a dose-dependent inhibition of 46mM K^+-evoked release of glutamate from rat cerebrocortical slices, with an IC_{50} of 20.72μM. Data are mean±sem (n=5-7). * illustrates statistical significance compared with control and $p<0.05$ by Kruskal Wallis.



Figs 6.7. Thiopentone at 300μM (A) inhibited 46mM K^+-evoked glutamate release by 55.4±16.8%. Thiopentone at 3μM (B) was ineffective. Data are mean±sem (n=5).

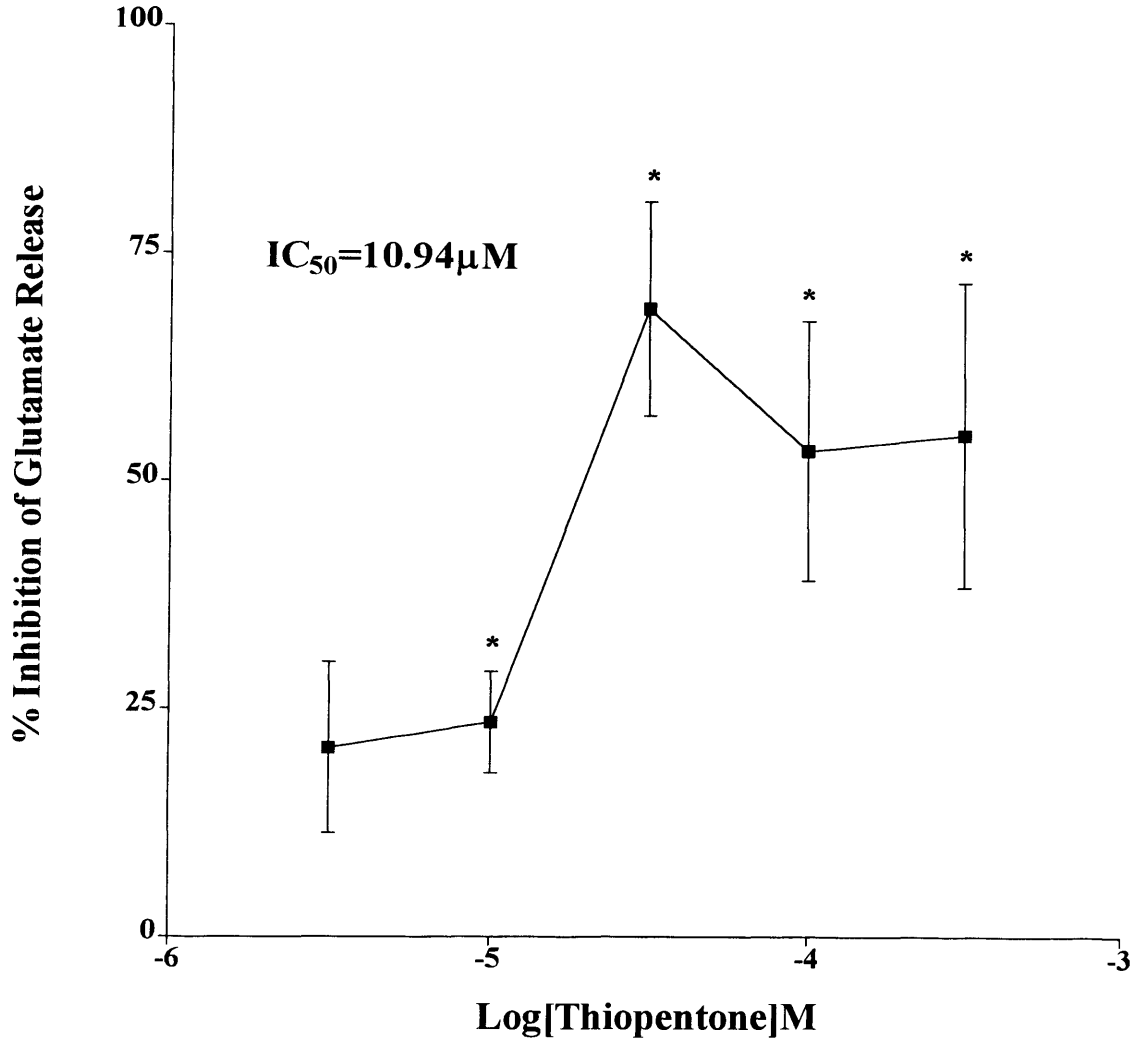
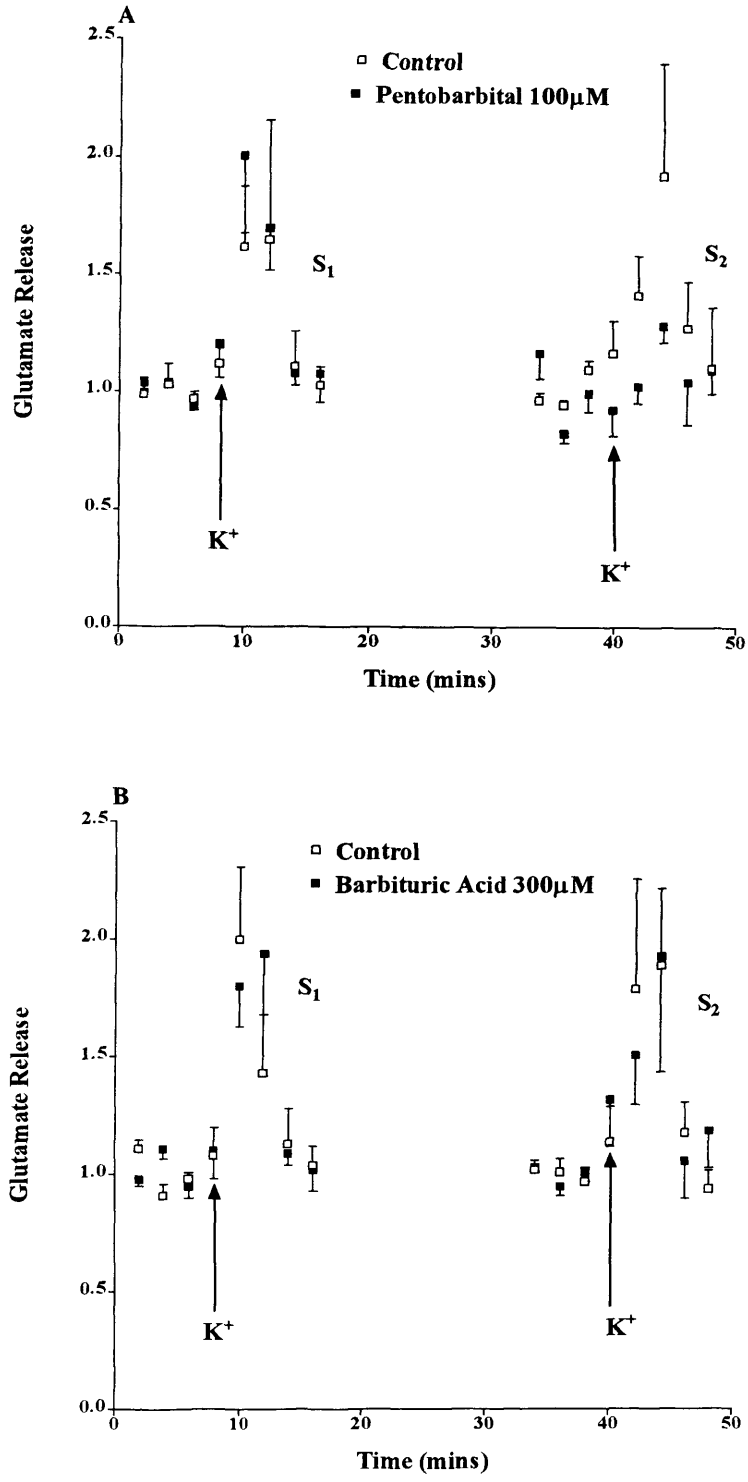


Fig 6.8. Thiopentone produced a dose-dependent inhibition of 46mM K^+-evoked release of glutamate from rat cerebrocortical slices, with an IC_{50} of 10.94μM. Data are mean±sem (n=5). * illustrates statistical significance compared with control and $p<0.05$ by Kruskal Wallis.



Figs 6.9. Time course of the effects of pentobarbital (100μM) (A) and barbituric acid (300μM) (B) on 46mM K^+-evoked release of glutamate from rat cerebrocortical slices. A significant inhibition of 60.4±14.4% was achieved with pentobarbital. Barbituric acid was ineffective. Data are mean±sem (n=5).

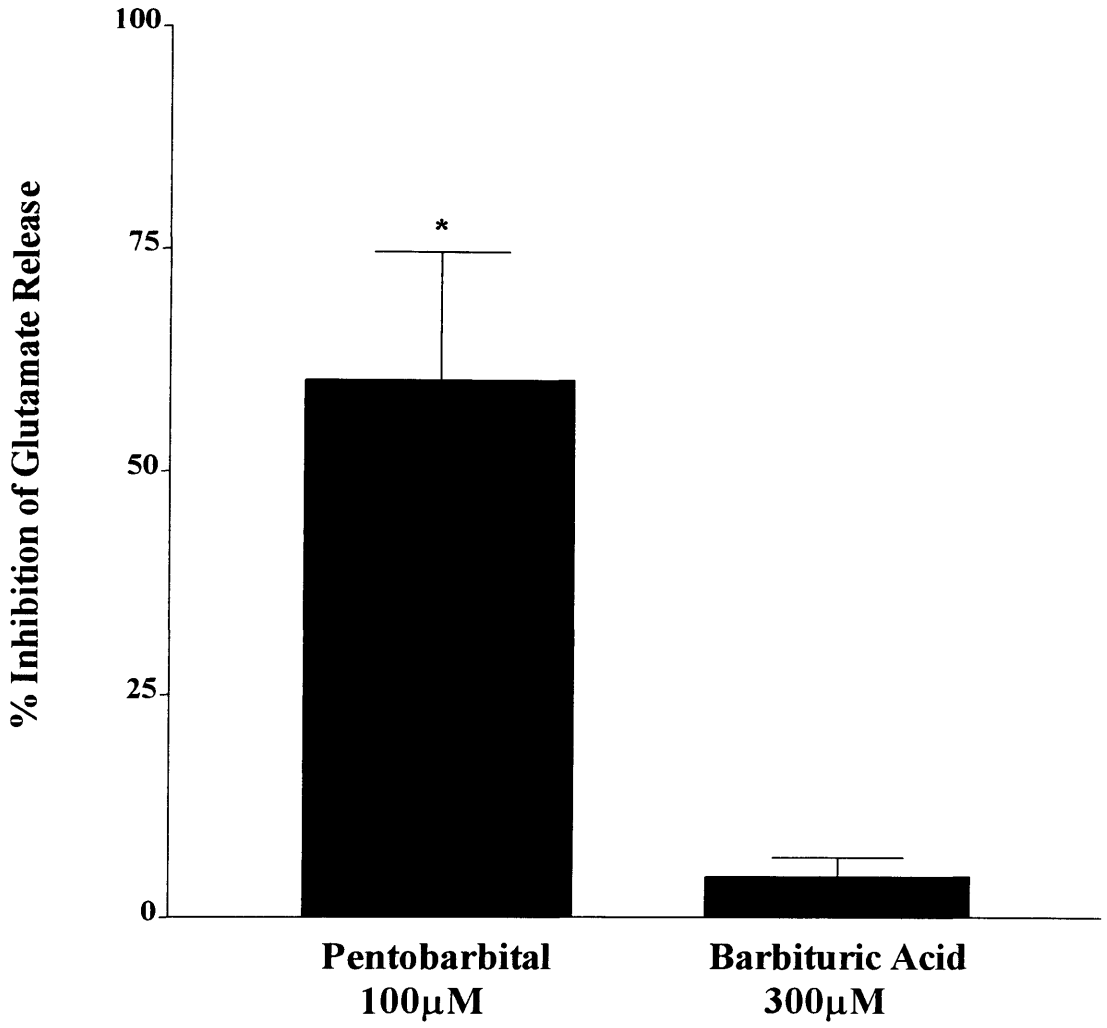


Fig 6.10. Pentobarbital (100μM), but not barbituric acid (300μM), significantly inhibited 46mM K^+-evoked release of glutamate from rat cerebrocortical slices. Data are mean±sem (n=5). * illustrates statistical significance compared with control.

Equilibration Times and Concentrations of Volatile Anaesthetics in the Perfusion Buffer Reservoir

Halothane at vaporiser settings of 1, 3 and 5% reached equilibrium within the perfusion buffer reservoir within 30min as illustrated in Table 6.1, therefore the time to equilibrium for 0.5, 7.5 and 10% was set at 30min. Isoflurane at 5% reached equilibrium within the reservoir after 30min (see Table 6.3). There was a significant decrease in volatile anaesthetic concentration when sampled at points A, B and C (see Figure 6.1), and more isoflurane was lost than halothane implying that isoflurane is more 'soluble' in plastic than halothane (Table 6.2 & 6.3).

Effect of Halothane and Isoflurane on Glutamate Release

Halothane produced a dose-dependent inhibition of K^+-evoked glutamate release from rat cerebrocortical slices (Figs 6.11 & 6.12) with an inhibition of 74.2±9.28% ($p<0.05$) achieved at 852±18.25μM (~2.87 Rat MAC) (Fig 6.11A). At 9.51±3.65μM (~0.03 Rat MAC) no inhibition was observed (Fig 6.11B). Further analysis of the dose-response curve yielded an estimated IC_{50} of 132μM (~0.44 Rat MAC).

Isoflurane at 435±15μM (1.24 Rat MAC) significantly inhibited the 46mM K^+-evoked release of glutamate from rat cerebrocortical slices, reducing the S_2/S_1 ratio by 64.00±7.28% compared with paired control (Fig 6.13).

Table 6.1. Time for halothane at vaporiser settings of 0.5, 1, 3, 5, 7.5 and 10% to reach equilibration within the bicarbonate buffer. 7.5 and 10% halothane vaporiser settings were achieved by linking two vaporisers in series. The aqueous concentration of 1% halothane at equilibrium is equivalent to 281μM [Franks and Lieb 1993]. Data are mean±sem for n=5.

	Halothane Vaporiser(s) Setting(s) (%)					
	0.5	**1**	**3**	**5**	**7.5**	**10**
Time (mins)	**Measured Concentrations in μM**					
5	-	305.67 ±13.96	501.88 ±09.19	1545.33 ±38.28	-	-
10	-	278.76 ±31.69	667.26 ±19.47	1474.33 ±61.05	-	-
15	-	347.68 ±39.41	889.65 ±19.30	1363.77 ±55.05	-	-
20	-	316.23 ±15.52	868.71 ±75.36	1441.09 ±77.75	-	-
30	75.19 ±5.10	316.9 ±1.18	864.26 ±3.67	1557.54 ±50.97	2687.98 ±26.12	3024.68 ±126.19
45	-	284.18 ±09.51	774.62 ±02.01	1475.41 ±19.72	-	-
60	-	306.15 ±03.99	742.01 ±30.66	1499.60 ±22.81	-	-

Table 6.2. Concentrations of halothane after an equilibration time of 30mins within the buffer, at various stages of the perfusion apparatus. Point A, B and C are: the tubing exiting the pump, the tubing immediately before entering the chamber, and within the chamber, respectively (Fig 6.1). Data are mean±sem for n=5.

	Halothane Vaporiser(s) Setting(s) (%)					
	0.5	**1**	**3**	**5**	**7.5**	**10**
Point	**Measured to the Concentrations in μM**					
A	-	-	-	576.30 ±34.42	-	-
B	-	-	-	361.09 ±8.13	-	-
C	9.51 ±3.65	85.25 ±5.74	222.51 ±3.80	395.65 ±16.42	670.61 ±19.50	852.88 ±52.3

Table 6.3. Time for isoflurane at vaporiser setting of 5% to reach equilibrium within the bicarbonate buffer (reservoir), and the concentration of isoflurane sampled from Point C (exiting the perfusion chamber) after 30min of equilibration. Data are mean±sem for n=5. Isoflurane (1%) at equilibrium corresponds to 240μM [Franks and Lieb 1993].

Time to Equilibration (min)	**Isoflurane Vaporiser Setting of 5% Measured Concentrations in μM**
15	1020.91±11.19
30	1147.70±29.16
45	1030.52±23.41
60	1025.69±23.25
Point C (sampled after 30min)	435.04±15.33

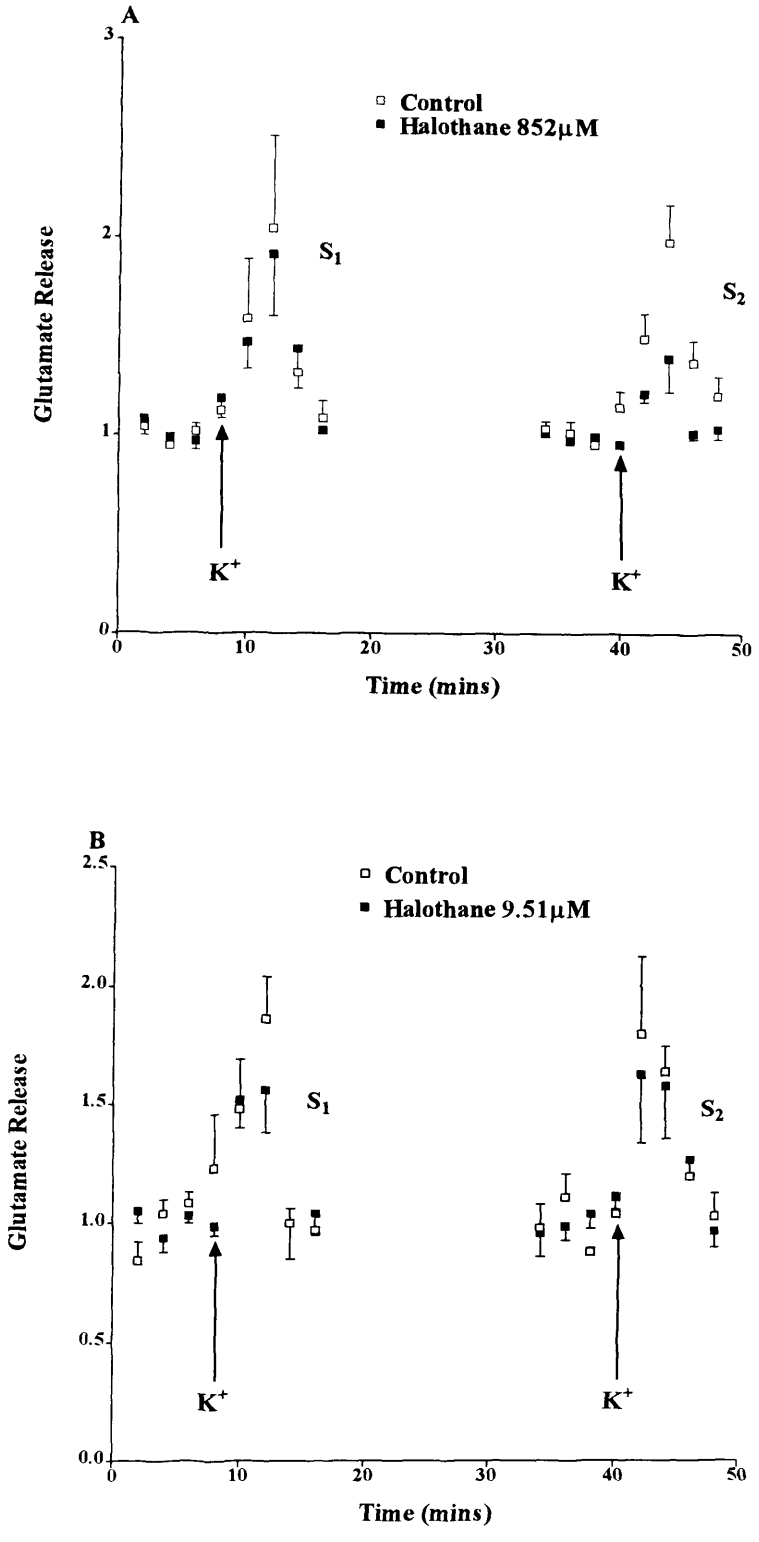


Fig 6.11. Halothane at 852.88±52.3μM (A) inhibited 46mM K^+-evoked release of glutamate from rat cerebrocortical slices by 74.2±10.5%. Halothane at 9.51±3.65μM (B) was ineffective. Data are mean±sem (n=6).

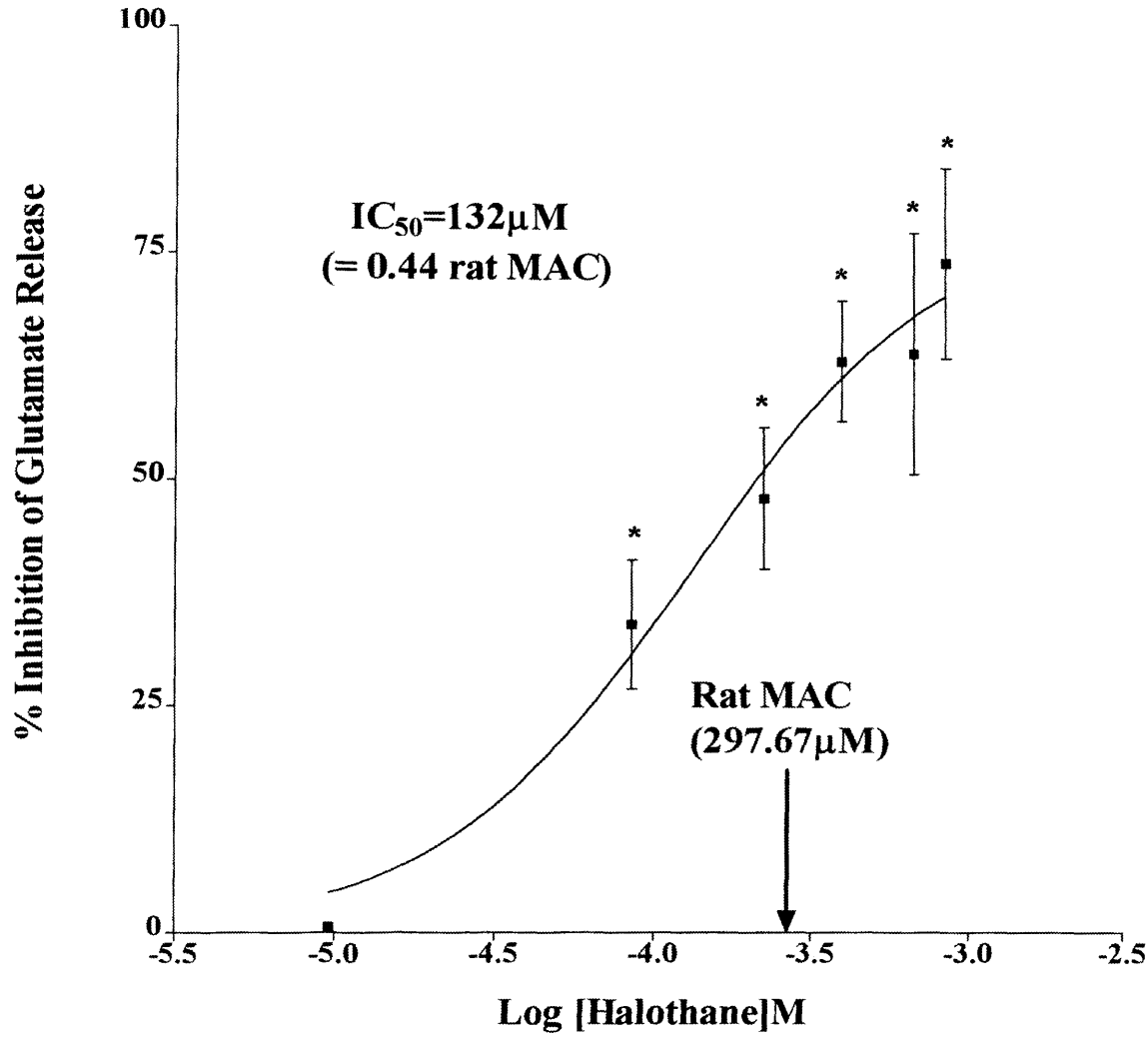


Fig 6.12. Halothane dose-dependently inhibited 46mM K^+-evoked release of glutamate from rat cerebrocortical slices, with an estimated IC_{50} of 132μM. Data are mean±sem (n=5-6). * illustrates statistical significance compared with control and $p<0.05$ by Kruskal Wallis.

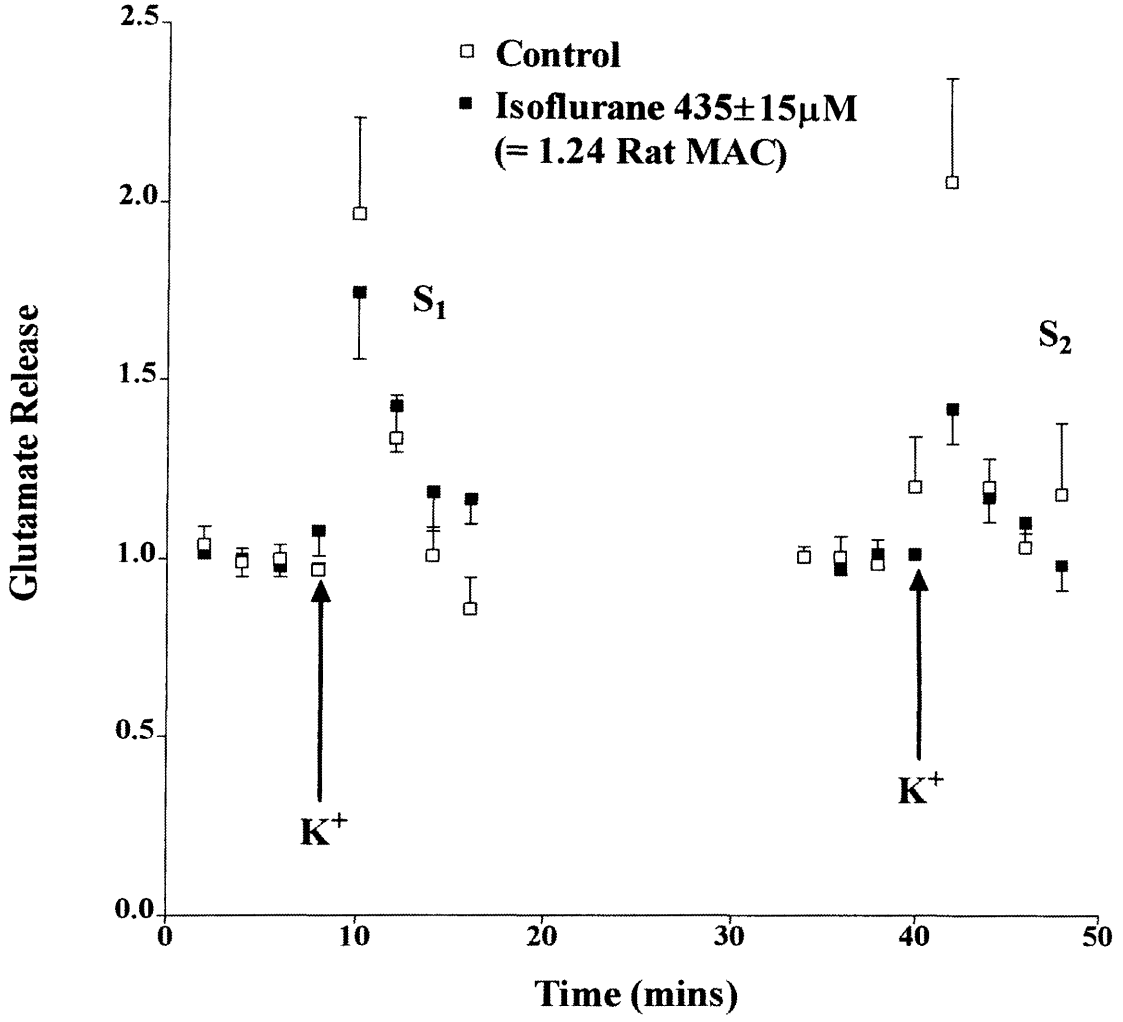


Fig 6.13. Isoflurane at 435.04±15.33μM significantly inhibited 46mM K^+-evoked glutamate release from rat cerebrocortical slices. A 64.00±7.28% reduction of S_2/S_1 ratios was observed. Data are mean±sem (n=5).

6.4. Conclusions

The data from this chapter illustrates that ketamine, propofol, thiopentone and halothane produce a dose-dependent inhibition of 46mM K^+-evoked glutamate release with estimated IC_{50} and I_{max} values of 18.2μM and 65%, 20.7μM and 75%, 7.6μM and 55%, and 132μM and 74% respectively. Full dose-response curves for the anaesthetic agents pentobarbital and isoflurane were not condstructed, but at single doses pentobarbital (100μM) and isoflurane (435μM) inhibited the K^+-evoked release by 60.4 and 64.0% respectively, while barbituric acid (300μM) was ineffective.

Total peak serum concentrations of ketamine, propofol, thiopentone and pentobarbital are in the range 20, 35, 380 and 15μM [Frenkel et al., 1993]. The free serum concentrations of these agents are 10-16, 1.05, 76-152 and 20-40μM when subtracting the proportion of the anaesthetic agent bound to plasma proteins (see Table 6.4). The inhibition of 46mM K^+-evoked glutamate release from rat cerebrocortical slices achieved in this study by ketamine and thiopentone (IC_{50}=18.2 and 10.94μM) and 100μM pentobarbital has proven to be clinically relevant when considering their respective protein binding [Reilly 1994]. The IC_{50} for propofol is 20 times the free serum concentration, however it has been reported that following an iv infusion of propofol the concentration of propofol in whole brain was 8.5 times that in plasma [Shyr et al., 1995]. Therefore free serum concentration of propofol could be as much as 10μM, and as the IC_{50} for propofol in inhibiting evoked release of glutamate is 20μM the top end of the clinical range (see Table 6.4).

There are many studies examining the effect of ketamine on NMDA receptors and glutamate release, the majority of which report that ketamine decreases glutamate release. Carla and Moroni in 1992 measured the DC potential between mouse cortical wedges following the application of NMDA and AMPA, with and without anaesthetic agents. Ketamine was reported to antagonise the NMDA response alone in a highly selective manner. The effect produced by a 2min pulse of AMPA was antagonised by halothane. Thiopentone and isoflurane both antagonised the effects produced either by AMPA or NMDA. In addition, Brockmeyer and Kendig 1995 examined the effects of ketamine on amino acid-mediated pathways in superfused neonatal rat spinal cord, concluding that

ketamine had a selective dose-dependent inhibitory effect on the ventral root potential, an NMDA receptor mediated event. Importantly they also illustrate that ketamine has no effect on the dorsal root potential, a GABA mediated event. Whilst it is clear that ketamine produces anaesthesia and this may result from a decrease of glutamate release, ketamine is not used clinically for neuroprotection (via an inhibition of excitotoxicity, Chapter 1 Section 1.6, page 15) as it has been shown to increase intracranial pressure, cerebral blood flow and the cerebral metabolic rate for oxygen [Jantzen 1994].

Table 6.4. A comparison of the IC_{50} values and the free serum concentrations for ketamine, propofol, thiopentone and pentobarbital [Frenkel et al., 1993]. [1] IV induction, [2] TIVA, [3] IV long term sedation (to my knowledge pentobarbitone is not used as an induction agent) and [4] IV induction.

Agent	% Protein Bound	Peak Serum Concentration (μM)	
		Total	Free
Thiopentone[1]	60-80	380	76-152
Propofol[2]	>97	35	1.05
Pentobarbitone[3]	60-80	200	40-80
Ketamine[4]	20-50	20	10-16

The effects of propofol on glutamate release remains unclear. Hans et al., 1994 reported that NMDA receptor-mediated toxicity was significantly reduced by the application of high concentrations of propofol (50-500μM) to cultured fetal rat hippocampal neurons. But Bickler et al., 1995, failed to inhibit the hypoxaemia-evoked glutamate release from rat cerebrocortical slices with propofol perhaps suggesting weak inhibition. More recently, propofol has been shown, at clinically relevant concentrations, to inhibit both the veratridine and 4-AP stimulated release of glutamate from rat cerebrocortical synaptosomes (IC_{50} = 30 and 39μM respectively), however no inhibition of K^+ stimulated release was seen [Ratnakumari and Hemmings 1997]. At variance with the general hypothesis by Larabee and Posternak (i.e. anaesthetics affect synaptic transmission and not axonal conduction), Ratnakumari and Hemmings showed that propofol significantly inhibited voltage-dependent Na^+ channels (IC_{50} = 46μM) by blocking veratridine-evoked

increases in $^{22}Na^{+}$ influx. Their results may indicate a role for neuronal voltage-dependent Na^{+} channels as a molecular target for general anaesthetic agents.

Bickler et al., 1995 reported that halothane and thiopentone decreased hypoxaemia-evoked glutamate release by 50-70%. Thiopentone and pentobarbital but not barbituric acid have been shown in this thesis to inhibit K^{+}-evoked glutamate release from rat cerebrocortical slices. The inhibition achieved may be due to the anaesthetic properties of thiopentone and pentobarbital, and not merely due to barbiturate structure as the non-anaesthetic barbiturate, barbituric acid was ineffective. Barbiturates have been shown potentiate the affinity of GABA to $GABA_A$ dependent on the presence of Cl^{-} ions [Ashton 1983]. Scatchard analysis of binding data indicates that barbiturates appear to increase the number of available GABA receptors and slow down the dissociation of GABA [Asano and Ogasawara 1991; Willow and Johnston 1991].

Schlame and Hemmings 1995, examined the effect of halothane on basal and evoked (4-AP, veratridine, increased KCl or ionomycin) release of glutamate from isolated rat cerebrocortical synaptosomes. Halothane was found to inhibit the 4-AP- and veratridine-, but not the basal, K^{+}-, or ionomycin-evoked glutamate release. In addition halothane inhibited the 4-AP- and K^{+}-evoked increase in free $[Ca^{2+}]_i$. Neither isoflurane, enflurane, nor halothane altered the basal release of glutamate from rat cerebrocortical synaptosomes but these agents were found to decrease the K^{+}-evoked glutamate release and K^{+}-evoked increase in $[Ca^{2+}]_i$ [Miao et al., 1995]. The decrease in K^{+}-evoked $[Ca^{2+}]_i$ was consistent with an inhibition of Ca^{2+} entry, possibly by specific voltage gated neuronal Ca^{2+} channels. Isoflurane and halothane have also been reported to depress glutamate mediated synaptic transmission [Maciver et al., 1996]. Maciver and colleagues stated that the increases observed in synaptic facilitation indicate presynaptic sites of action that may contribute to anaesthetic-induced depression of excitatory transmission.

An inhibition of glutamate release might contribute to anaesthesia, however an enhancement of GABA action and inhibition of Ca^{2+} channels are the other possible sites. Clinically relevant concentrations of anaesthetics have been reported to enhance GABAergic inhibition by two primary actions: an increase in GABA affinity for the

$GABA_A$ receptor and prolongation or augmentation of the Cl^-conductance [Tanelian et al., 1993]. Halothane, enflurane, ether and methoxyflurane have been reported to increase the uptake of radiolabelled Cl^- in a concentration dependent, picrotoxin-sensitive manner [Moody et al., 1988]. This study also demonstrates the volatile anaesthetic inhibition of TBPS (non-competitive $GABA_A$ antagonist) binding to the same preparation, but at supra-clinical concentrations. A concentration dependent reduction in the binding of TBPS and an enhancement of the uptake of Cl^- was also observed in the presence of clinically relevant concentrations of propofol [Peduto et al., 1991]. An increase of GABA in the transmitter pool of rat cerebrocortical slices has been reported to occur in the presence of 3% halothane, this was not due to an inhibition of uptake but to catabolism [Cheng and Brunner 1975 & 1981]. However at varience with the 'GABA' hypothesis is the reported inhibition of GABA release following pentobarbitone [Collins 1980].

Hirota and Lambert (1996A & B) examined the effect of I.V. anaesthetic agents on the binding of dihydropyridine to L-type voltage sensitive Ca^{2+} channels in rat cerebrocortical membranes. Thiopentone and pentobarbitone, but not barbituric acid interacted and consequently inhibited the binding, suggesting that the anaesthesia achieved with both thiopentone and pentobarbitone may be due, in part, to the inhibition of L-type voltage sensitive Ca^{2+} channels.

It is clear that a unitary theory of anaesthetic action cannot account for the effects observed with all anaesthetic agents. The actions seen at glutamatergic transmission/glutamate receptors, the $GABA_A$ receptor complex, and Ca^{2+} channels would make a multisite theory of anaesthesia likely.

Chapter 7. Effects of Intravenous and Inhalation Anaesthetic Agents and Morphine on the Binding Properties of [^{3}H]MK-801 to Rat Cerebrocortical Membranes

7.1. Introduction

When a typical glutamate-containing neurone fires, an action potential (AP) is propagated down the axon. The AP depolarisation increases the membrane potential, opening sodium channels and increasing the membrane permeability to sodium ions several hundredfold. As there is more Na^+ entering the cell the membrane potential becomes less negative and reverses polarity. Following the closure of Na^+ channels, K^+ channels open resulting in repolarisation. The voltage change produced by the movement of Na^+ and K^+ ions activates voltage sensitive calcium channels (VSCCs), allowing Ca^{2+} to enter the cell. Increases in intracellular Ca^{2+} result in the exocytotic release of vesicular glutamate from pre-synaptic terminals [Nicholls and Attwell 1991]. The released glutamate can then pass across the synaptic cleft where it can bind to glutamate receptors located on the post-synaptic terminal and also feedback onto glutamate receptors located on the presynaptic terminal, to enhance glutamate release (see Fig 7.1).

The NMDA receptor contains an integral cationic channel, that is highly permeable to Ca^{2+}, Na^+ and K^+. It also has several modulatory domains in addition to the primary glutamate/NMDA recognition site: i) a divalent cation binding site within the channel pore, to which Mg^{2+} ions bind, ii) a binding site recognised by dissociative anaesthetics (ketamine and phencyclidine) and MK-801, within the channel, both sites are inhibitory and iii) modulatory sites, sensitive to glycine, Zn^{2+} and polyamines [Teichberg 1992; Scatton 1993]. The Mg^{2+} block of the NMDA receptor, only occurs when the neurone is at rest. Following depolarisation the Mg^{2+} block is removed allowing the movement of Ca^{2+} and Na^+ into the neurone. Any occupation of the binding site recognised by dissociative anaesthetics and MK-801, inhibits glutamate or NMDA stimulated ionic currents and these agents act as non-competitive antagonists at this receptor [Kemp et al., 1987]. As NMDA receptors have also been located on the presynaptic terminal of excitatory synapses [Conti

et al.,1997; DeBiasi et al., 1996], activation of these receptors would result in a positive feedback loop evoking more release.

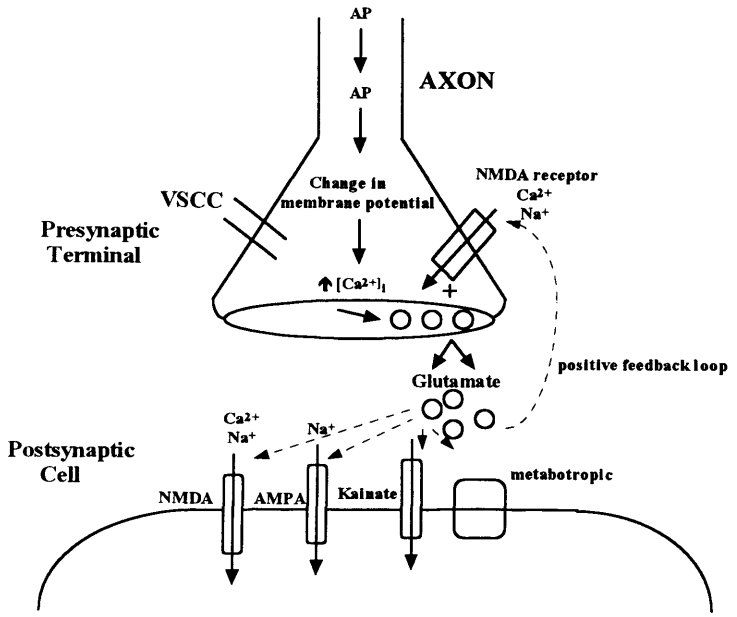
This chapter will investigate the interaction of anaesthetic agents with the binding site for [^{3}H]MK-801 to NMDA receptors. This binding site is reconised as that for dissociative anaesthetics. A brief discussion of radioligand binding is briefly presented. The principle of radioligand binding assays is to enable the properties of drug-receptor interaction to be studied. From a saturation assay, where the concentration of radiolabel used is increased until saturation is reached, the parameters K_d (equilibrium dissociation constant, i.e. the concentration of radioligand required to produce 50% B_{max}) and B_{max} (amount of specific binding at saturation) can be estimated. Analysis of saturation binding assays can be performed by one of three methods; a rectangular hyperbola, Hill analysis and Scatchard analysis.

Displacement curves enables the affinity of an unlabelled drug to be estimated. This is performed by labelling a known concentration of receptors with a fixed concentration of a radioactive compound and increasing the concentration of unlabelled compound to displace the radioactive compound. Following displacement of the radiolabel, the true affinity of that displacer for the receptor-K_i could be calculated. K_i is an estimate of the K_d for the unlabelled ligand. K_i values are the 50% inhibitory concentration (IC_{50}) of displacer, corrected for radiolabelled ligand concentration using the Cheng and Prusoff equation (1972):

$$K_i = IC_{50}/(1 + (L/K_d))$$

Where L is the concentration of radiolabel, and K_d the equilibrium dissociation constant of the radiolabel for the receptor. IC_{50} values are dependent on radioligand concentration. For example, if the concentration of radioligand is increased, a higher concentration of displacer is required in order to achieve the same amount of displacement. This has the effect of causing the displacement curves to shift to the right. The Cheng and Prussoff equation takes into account both the concentration and the affinity of the radioligand to shift the curve back to the left and predict K_i.

Fig 7.1. Schematic diagram of exocytotic glutamate release.



Aims

Based on the data accumulated from Chapter 6 of this thesis, several intravenous and inhalation anaesthetic agents have been shown to inhibit 46mM K^+-evoked glutamate release from rat cerebrocortical slices. The mechanism of inhibition is at this stage unclear however, some reports suggest that anaesthetic agents can: depress glutamate transmission via presynaptic receptor actions [Maciver et al., 1996], enhance $GABA_A$ receptor mediated transmission [Tanelian et al., 1993], or inhibit Ca^{2+} channels [Hirota and Lambert 1996A & B]. NMDA receptors have been located presynaptically [DeBiasi et al., 1996; Conti et al., 1997], activation of which could possibly lead to an enhancement of glutamate release via a positive feedback loop. An inhibition of glutamate release was observed in Chapter 6, which may be due to NMDA receptor blockade.

Using cerebrocortical membranes which possess NMDA receptors, the binding properties of [^{3}H]MK-801 to NMDA receptors will be studied to probe the interaction of anaesthetic agents with both pre- and postsynaptic NMDA glutamate receptors. If any anaesthetic agent interferes with the binding of [^{3}H]MK-801, it may implicate NMDA receptors in the control of the positive feedback of glutamate and glutamate release, and possibly in the mechanism of anaesthesia.

7.2. Methods

Radioligand Binding Assays

Cerebrocortical membranes used in radioligand binding assays were prepared in HEPES-EDTA buffer (HEPES 10mmol litre^{-1} and EDTA 1mmol litre^{-1}, pH 7.4 with 10M NaOH), as described in Chapter 2, Section 2.5, page 43. Membrane protein concentration was estimated by the method of Lowry et al., 1951, and adjusted to achieve a protein concentration of 200μg in each tube, unless otherwise stated. Receptor binding was determined indirectly as the radioligand binds non-specifically to non-receptor protein. The binding assays consisted of membranes, 100μM glutamate, 30μM glycine and [^{3}H]MK-801 at 37°C in a final assay volume of 1ml. Non-specific binding was determined in the presence of unlabelled MK-801 at a final concentration of 30μM. The membranes were harvested onto Whatman GF/B glass fibre filters by rapid vacuum filtration using a Brandel Cell Harvester, and washed three times using 5ml HEPES-EDTA buffer each time. The radioactivity remaining on the filters was extracted using Optiphase "Safe" as a scintillant, and the activity quantified by liquid scintillation spectroscopy using a Packard 1900 TR Liquid Scintillation Analyser.

Time to Equilibrium

Binding was measured in the presence of [^{3}H]MK-801 at 2nM corresponding to the concentration used in a report by Gredal et al., 1996 at 37°C for increasing time intervals 5-90min (see Table 7.1).

Saturation Analysis

Saturation binding assays were performed using a range of [^{3}H]MK-801 concentrations approximately 0.19, 0.39, 0.78, 1.57, 3.13, 6.25, 12.5 and 25nM for 90min at 37°C (see Table 7.1). The binding parameters K_d (equilibrium dissociation, nM) and B_{max} (maximum number of binding sites, fmoles/mg protein) were estimated from specific binding curves using: rectangular hyperbola, Hill and Scatchard analysis.

Glutamate and Glycine Dependence

The dependence of glutamate and glycine on [^{3}H]MK-801 binding to rat cerebrocortical membranes was characterised. The binding assay was conducted in the presence of [^{3}H]MK-801 at a concentration of approximately 2nM obtained from the saturation analysis and was performed in the presence of both glutamate and glycine, glutamate alone, glycine alone and without glutamate or glycine. Assay volumes are as in Table 7.1, with the balance made up to 1ml from the assay buffer.

Table 7.1. Standard binding assay addition table. Volumes are in µl.

	Amount added (final volume of 1ml)					
	Membranes	**MK-801**	**Glutamate**	**Glycine**	**[^{3}H]MK -801**	**Buffer**
Total	100	0	200	200	200	300
NSB	100	200	200	200	200	100

Displacement Binding Assays

All displacement assays were performed in 1ml volumes as described in Table 7.2. The binding of ~3nM [^{3}H]MK-801 was displaced by 10nM-100mM Ketamine (*racemic*, *R*- and *S*+), 3nM-0.1mM barbituric acid, etomidate, propofol and thiopentone, 1-100µM pentobarbital and morphine. Incubations were for 90min at 37°C and were terminated as described previously. Isotopic dilutions of ~0.5nM [^{3}H]MK-801 with 0.3nM-10µM MK-801 were also performed as a displacement assay.

Table 7.2. Table of addition for the displacement of [^{3}H]MK-801 binding to rat cerebrocortical membranes. Ligand represents the cold displacing agent applied at a range of concentrations.

	Amount added (final volume of 1ml)						
	Membranes	**MK-801**	**Glutamate**	**Glycine**	**[^{3}H]MK-801**	**Ligand**	**Buffer**
Total	100	0	200	200	200	0	300
NSB	100	200	200	200	200	0	100
Ligand	100	0	200	200	200	200	100

Displacement Binding Assay with Halothane

The effects of halothane on the displacement of MK-801 were investigated using isotopic dilutions of [^{3}H]MK-801 with MK-801. The assay medium consisted of [^{3}H]MK-801, glutamate, glycine, buffer and MK-801 (concentration range determined from the displacement binding assay, and 30μM for NSB), and was pre-equilibrated for 15min at 37°C with halothane. Halothane was delivered using a precalibrated Fluotec 3 vaporiser (at vaporiser settings of 1 and 3%), with humidified carbogen (95% O_2/5% CO_2) at 2litres/min as the carrier gas. The delivery system consisted of a 12 port manifold, from which either halothane or carbogen were perfused through to the assay medium and the membranes. Following pre-equilibration, the cerebrocortical membranes were added and the reaction harvested after 45min. Halothane concentrations were measured using GC (Chapter 2, Section 2.10, page 46).

Assessment of Cultured Neuronal Preparations for NMDA Receptor Expression

As the use of brain membranes can provide limited information on the functional coupling of NMDA receptors, two cell lines previously reported to express these receptors were evaluated. SH-SY5Y human neuroblastoma [Nair et al., 1996] and murine P19 embryonal carcinoma [McBurney et al., 1988] cell lines were cultured and harvested as described in Chapter 2 Section 2.6 & 2.7, page 44. A saturation analysis using only the highest concentration of [^{3}H]MK-801 (~25nM) was performed on cell membranes derived from these cell lines in HEPES-EDTA buffer (HEPES 10mmol litre^{-1} and EDTA 1mmol litre^{-1}, pH 7.4 with 10M NaOH) in order to determine B_{max}. In addition the $[Ca^{2+}]_i$ in response to NMDA in SH-SY5Y and P19 cells was determined using fura-2, as described in Chapter 2 Section 2.9, page 45, in Krebs-HEPES buffer (NaCl 143, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$, 1.2, $CaCl_2$ 2.0, glucose 11.7 and HEPES 10mmol litre^{-1}, pH7.4).

Data and Statistical Analysis

Saturation data was analysed using a rectangular hyperbola (radioactivity bound (fmol/mg protein) vs [radioactive ligand](nM)), a Hill (radioactivity bound (fmol/mg protein) vs log[radioactive ligand]M) and a Scatchard (bound/free (fmol/mg protein/nM vs bound (fmol/mg protein)) analysis using Graphpad Prism 2. Data are presented as mean±sem (n).

Statistical analysis was performed using Kruskal Wallis and Wilcoxon rank sum tests where appropriate, and considered significant when $p<0.05$.

7.3. Results

The binding of [^{3}H]MK-801 to rat cerebrocortical membranes was time dependent reaching equilibrium at approximately 60min (Fig 7.2).

Saturation Analysis

The binding of [^{3}H]MK-801 was concentration dependent with B_{max} and K_d values shown in Table 7.3. At the radioligand K_d, NSB was ~ 15%. All data were first analysed by a one site rectangular hyperbola to ensure saturation of the radioligand, followed by Hill and Scatchard as displayed in Table 7.3. A graphical representation of one experiment analysed by a rectangular hyperbola (A), Hill (B) and a Scatchard (C) is illustrated in Fig 7.3. As there were no statistical differences between the K_d and B_{max} values obtained by the three methods of analysis, any further analysis of the binding characteristics of [^{3}H]MK-801 to rat cerebrocortical membranes was performed using a concentration of ~3nM [^{3}H]MK-801 (K_d), estimated via Scatchard analysis.

Table 7.3. B_{max} and K_d for [^{3}H]MK-801 binding to rat cerebrocortical membranes, analysed from saturation binding isotherms by one site rectangular hyperbola, Hill and Scatchard. * mean slope factor = 1.16±0.1. Data are expressed as mean±sem (n=6).

Method of Analysis	B_{max} (fmol/mg protein)	K_d (Hill = pK_d)
Rectangular Hyperbola	826±90	2.33±0.26nM
Hill Analysis*	809±101	8.68±0.09
Scatchard	910±86	2.87±0.35nM

Glutamate and Glycine Dependency

The binding of ~3nM [^{3}H]MK-801 to rat cerebrocortical membranes was dependent on the presence of glutamate. The binding was not significantly reduced if glycine alone was removed. Removal of glutamate alone significantly reduced the binding by 16% and removal of both glutamate and glycine significantly reduced the binding by 47%, compared to the binding in the presence of both glutamate and glycine (Fig 7.4).

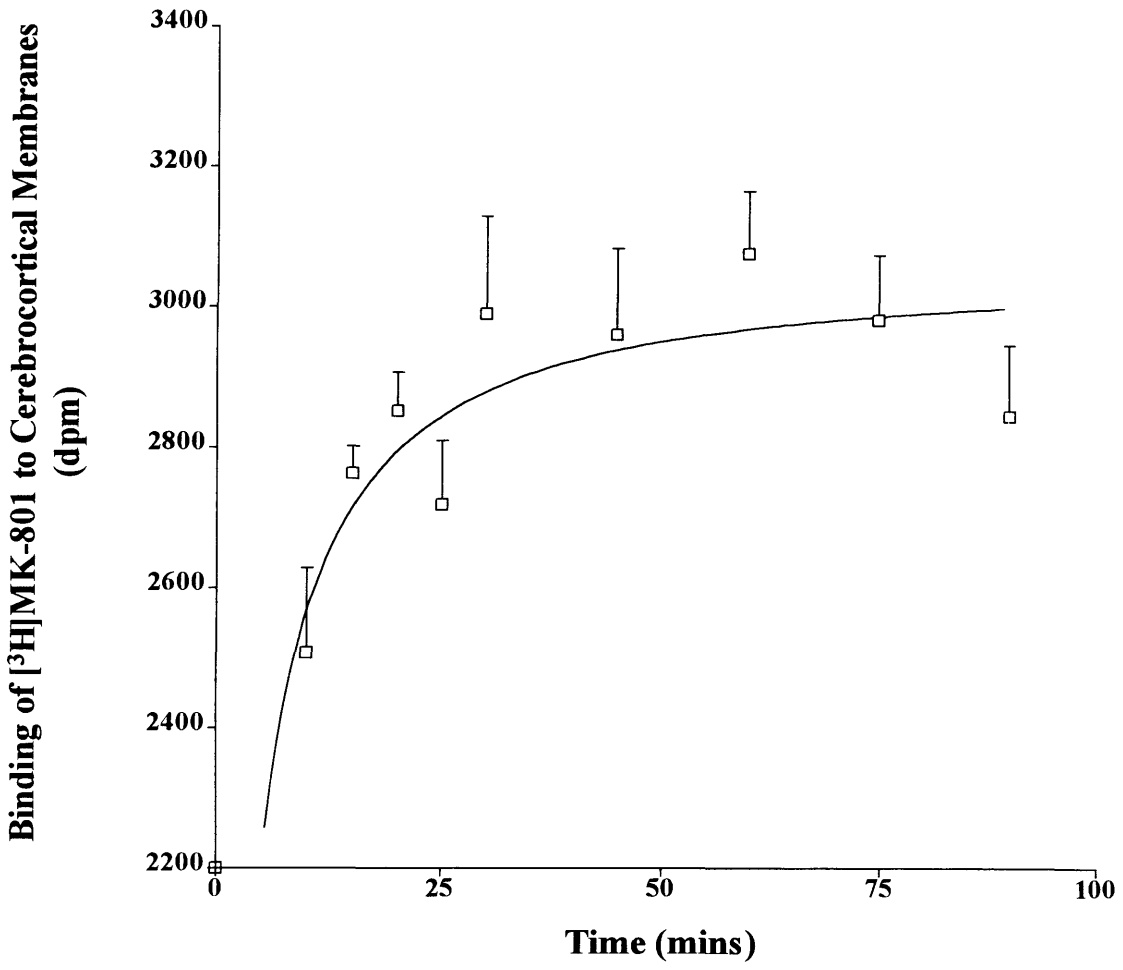


Fig 7.2. 2.88nM [^{3}H]MK-801 binds to cerebrocortical membranes in a time dependent fashion. The data is expressed as mean±sem (n=6).

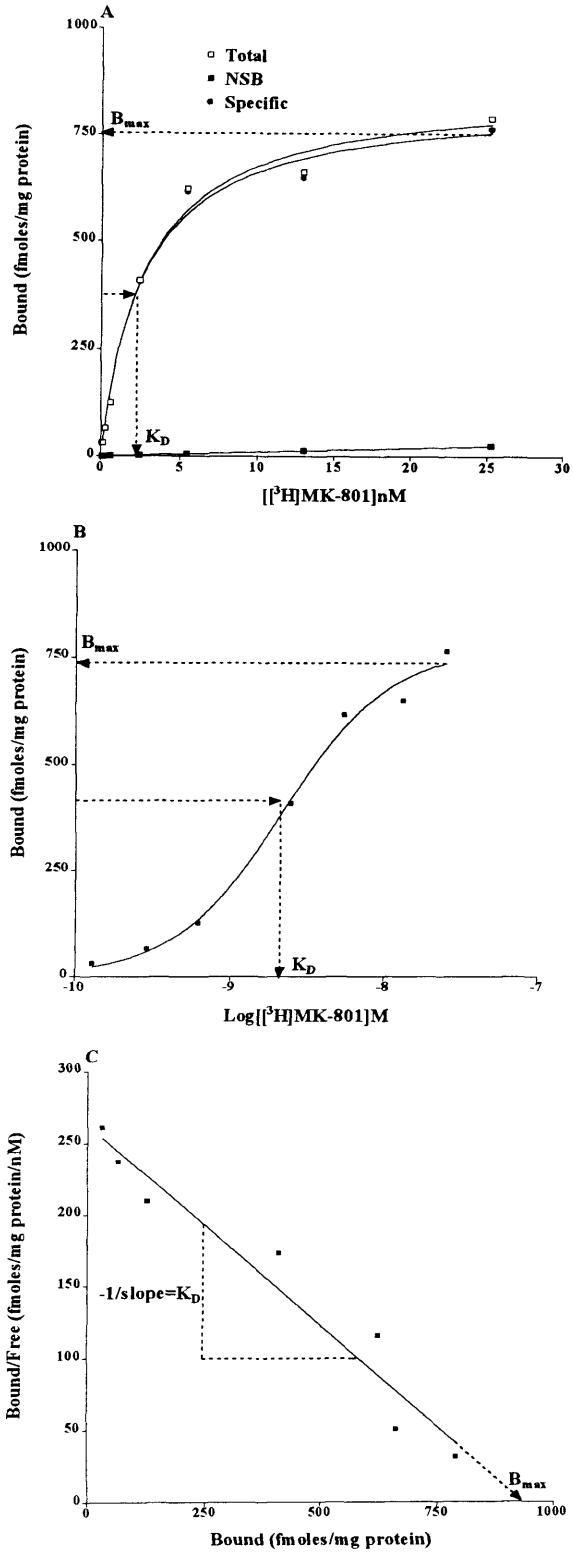


Fig 7.3. A, B and C are representative from 6 individual experiments of the saturation analysis of [^{3}H]MK-801 binding to rat cerebrocortical membranes. A is a one site rectangular hyperbola, B is a Hill and C is a Scatchard.

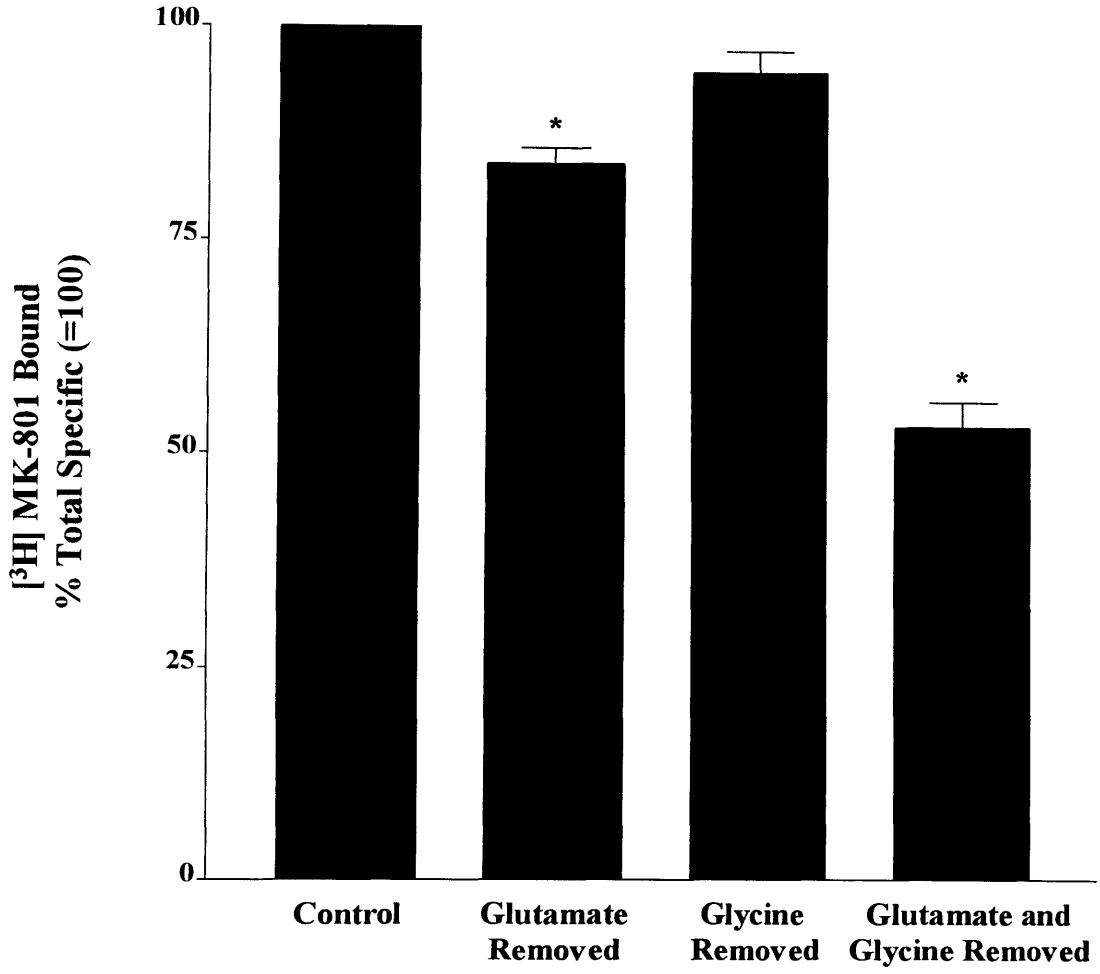


Fig 7.4. Removal of either glutamate alone or both glutamate and glycine together significantly reduced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes. Data are mean±sem (n=6). * illustrates statistical significance compared with binding in the presence of both glutamate and glycine.

Effect of Intravenous Anaesthetic Agents, Barbituric Acid, Morphine and Halothane

The *racemic* mixture, *R*- and the *S*+ isomer of ketamine displaced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes (Fig 7.5 and Table 7.3). MK-801 also displaced with a $pK_i(K_i)$ of 8.65 (2.22nM) ±0.14 (Fig 7.6) for comparison and to confirm the K_d determined directly.

Barbituric acid (A), propofol (B) and thiopentone (C) (3nM-0.1mM), pentobarbital (D) and morphine (E) (1-100μM) failed to displace [^{3}H]MK-801 binding to rat cerebrocortical membranes (Fig 7.7). Etomidate (F) displaced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes with an estimated $pK_i(K_i(\mu M))$ of 5.40(4.01)±0.02 (Fig 7.7). Halothane failed to significantly affect the displacement of [^{3}H]-MK-801 binding with MK-801 (Fig 7.8 and Table 7.4).

Table 7.3. The mean $pK_i(K_i)$ and slope factors for the displacement of [^{3}H]MK-801 binding with ketamine (*rac*, *R*- and *S*+). Data are mean±sem (n=6).

Agent	$pK_i(K_i(nM))$	Slope Factors
Ketamine (*rac*)	6.20±0.04(638.26)	0.85
Ketamine (*R*-)	5.57±0.08(2703.96)	0.98
Ketamine (*S*+)	6.25±0.04(568.85)	1.15

Table 7.4. The mean $pK_i(K_i(nM))$ and maximum number of binding sites in the presence of halothane and the carrier gas for halothane. Data are mean±sem (n=4), ns=no significant difference.

	$pK_i(K_i(nM))$		Amount of [^{3}H]MK-801 Bound (fmoles/mg protein)	
Concentration of Halothane (μM)	**Carrier Gas**	**Halothane**	**Carrier Gas**	**Halothane**
313.22±27.37	8.54(2.88)±0.11	9.12(0.76)±0.22^{ns}	51.2±8.41	40.40±5.06^{ns}
879.18±35.11	8.82(1.51)±0.18	9.07(0.85)±0.34^{ns}	64.96±10.28	49.95±12.4^{ns}

Assessment of NMDA receptors on SH-SY5Y and P19 Cell Lines

1. Binding

The total binding in the presence of ~20nM [^{3}H]MK-801 for SHSY-5Y cells was 4967.21±259.15dpm compared to the NSB of 4113.98±170.66dpm. The total binding in the presence of 20nM [^{3}H]MK-801 for P-19 cells was 4012.62±193.65dpm compared to the NSB of 3267.71±214.31dpm. These data suggest extremely low levels of expression.

2. Fluorimetry

$[Ca^{2+}]_i$ in SH-SY5Y cells did not increase following the addition of 100 and 500μM NMDA either with or without Mg^{2+} in the buffer. Following the addition of 20μM carbachol an increase of ~360nM of $[Ca^{2+}]_i$ was observed (Fig 7.9A), confirming the integrity of the cell preparation. The application of 200μM NMDA twice to undifferentiated P19 cells did not increase $[Ca^{2+}]_i$ (Fig 7.9B). In addition a positive control could not be found as the application of 46mM K^+ and 1mM carbachol to undifferenicated P-19 cells did not produce an increase in $[Ca^{2+}]_i$. P19 cells differentiated for 2 weeks spontaneously lysed during the 20min de-esterification at room temperature, therefore the effect of NMDA application could not be studied.

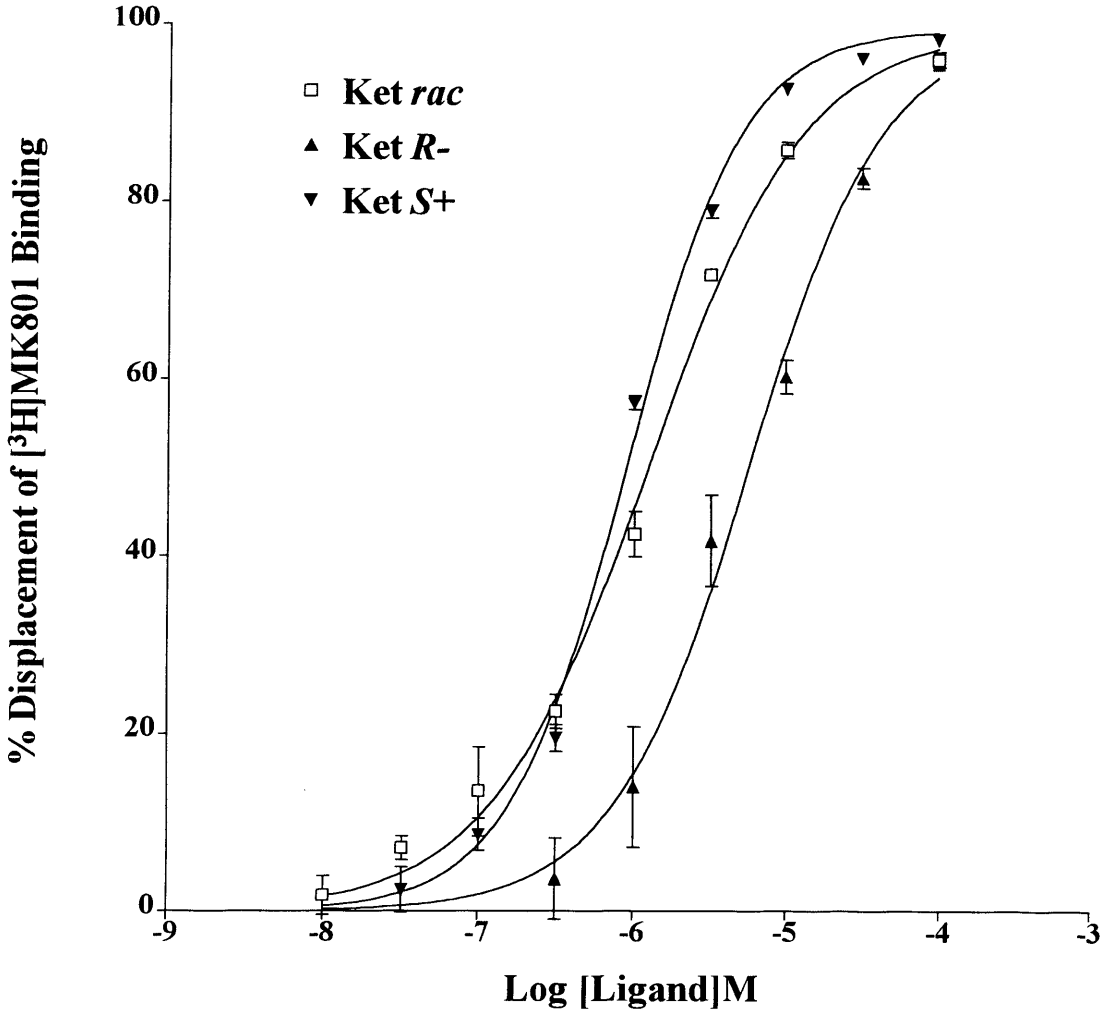


Fig 7.5. Ketamine, *rac*, *R*- and *S*+ displace [^{3}H]MK-801 binding with estimated pK_i's of 6.2, 5.6 and 6.25. Data are mean±sem (n=6).

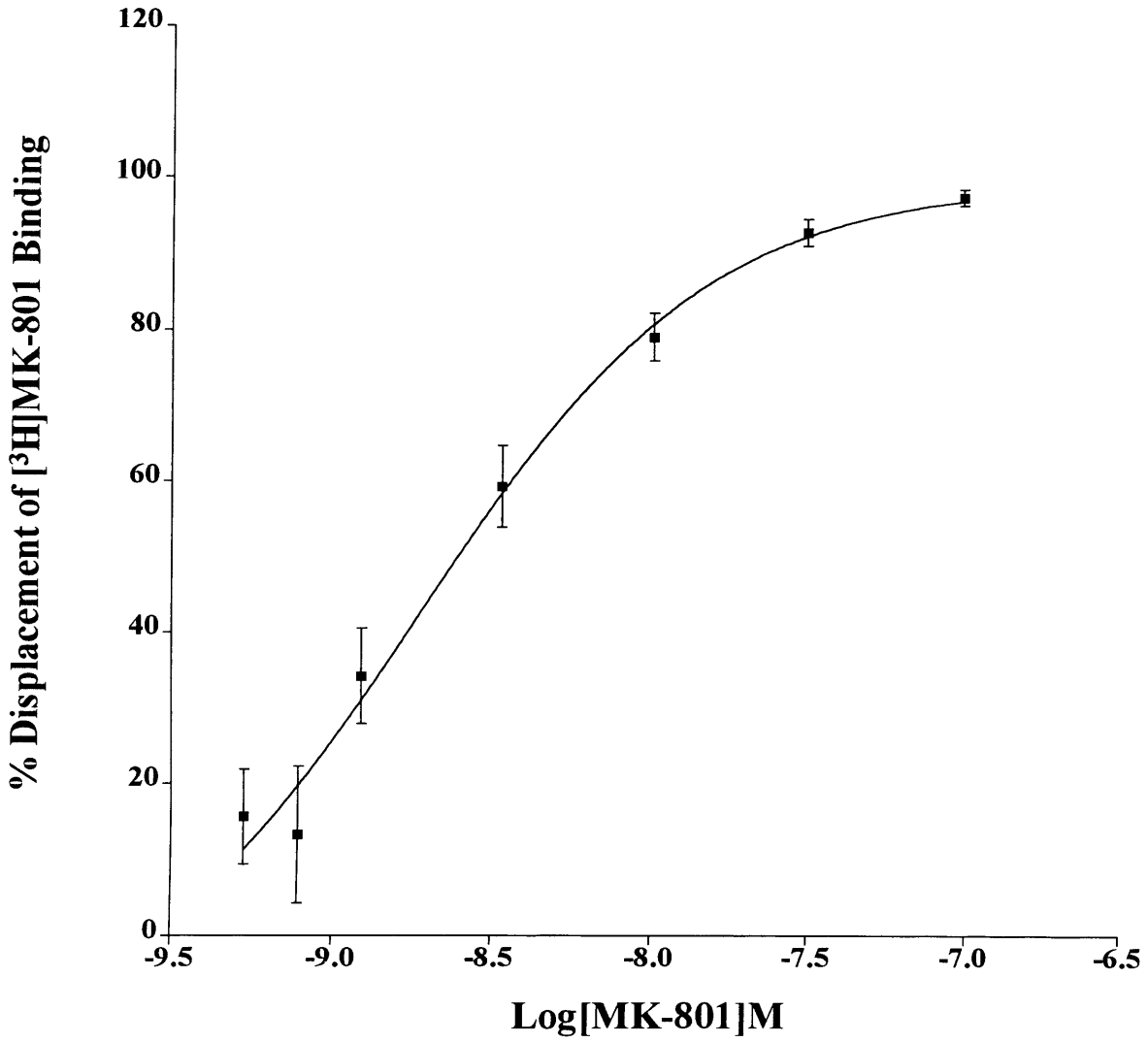


Fig 7.5. MK-801 displaces the binding of [^{3}H]MK-801 to rat cerebrocortical membranes with an estimated pK_d of 8.65±0.14. Data are mean±sem (n=15).

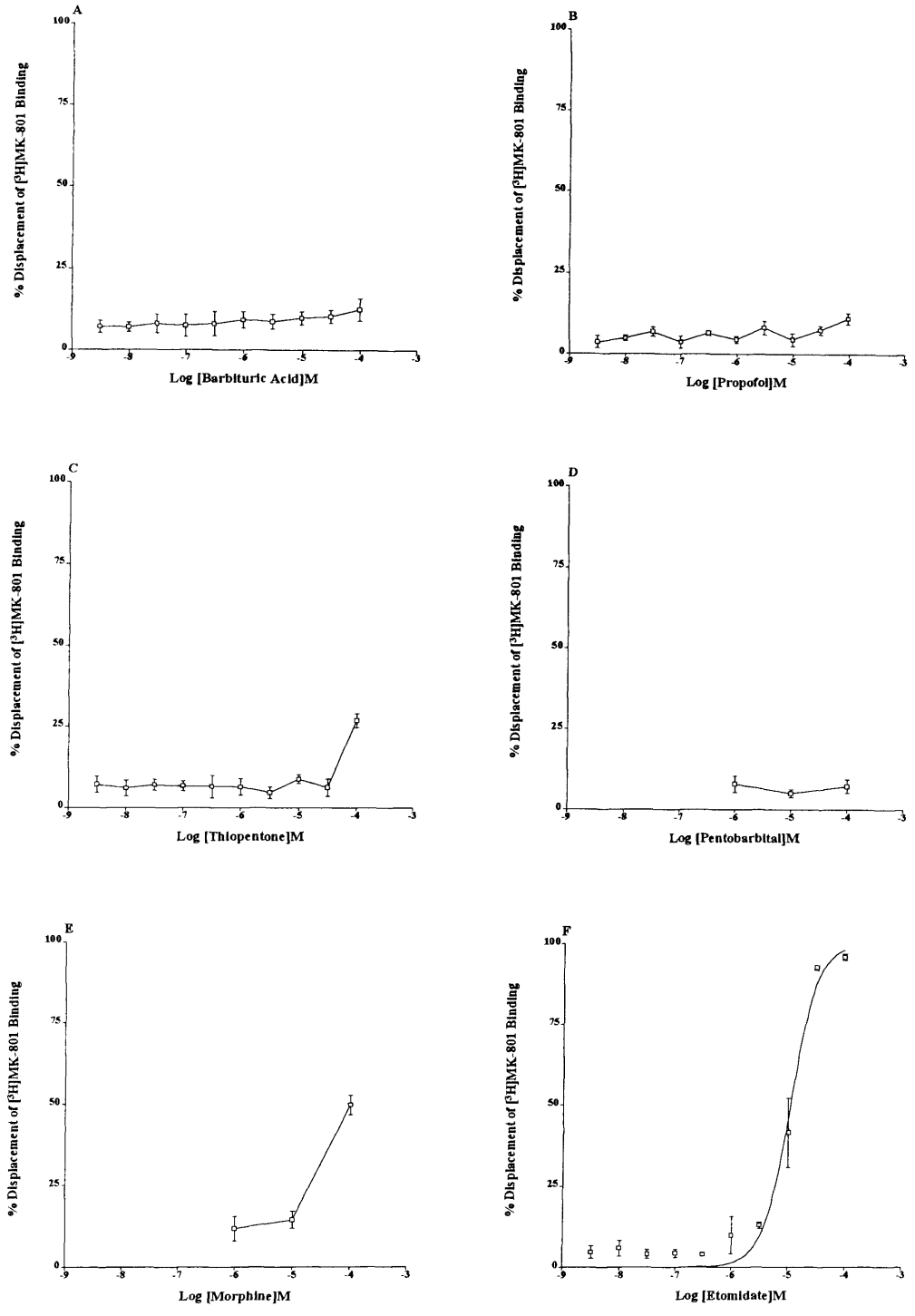


Fig 7.7. Barbituric acid (A), propofol (B), thiopentone (C), pentobarbitone (D) and morphine (E) failed to displace the binding of [^{3}H]MK-801 to rat cerebrocortical membranes at clinically relevant concentrations. Etomidate (F) displaced with an estimated $pK_i(K_i)$ of 5.40(4.01μM)±0.02. Data are mean±sem (n=6).

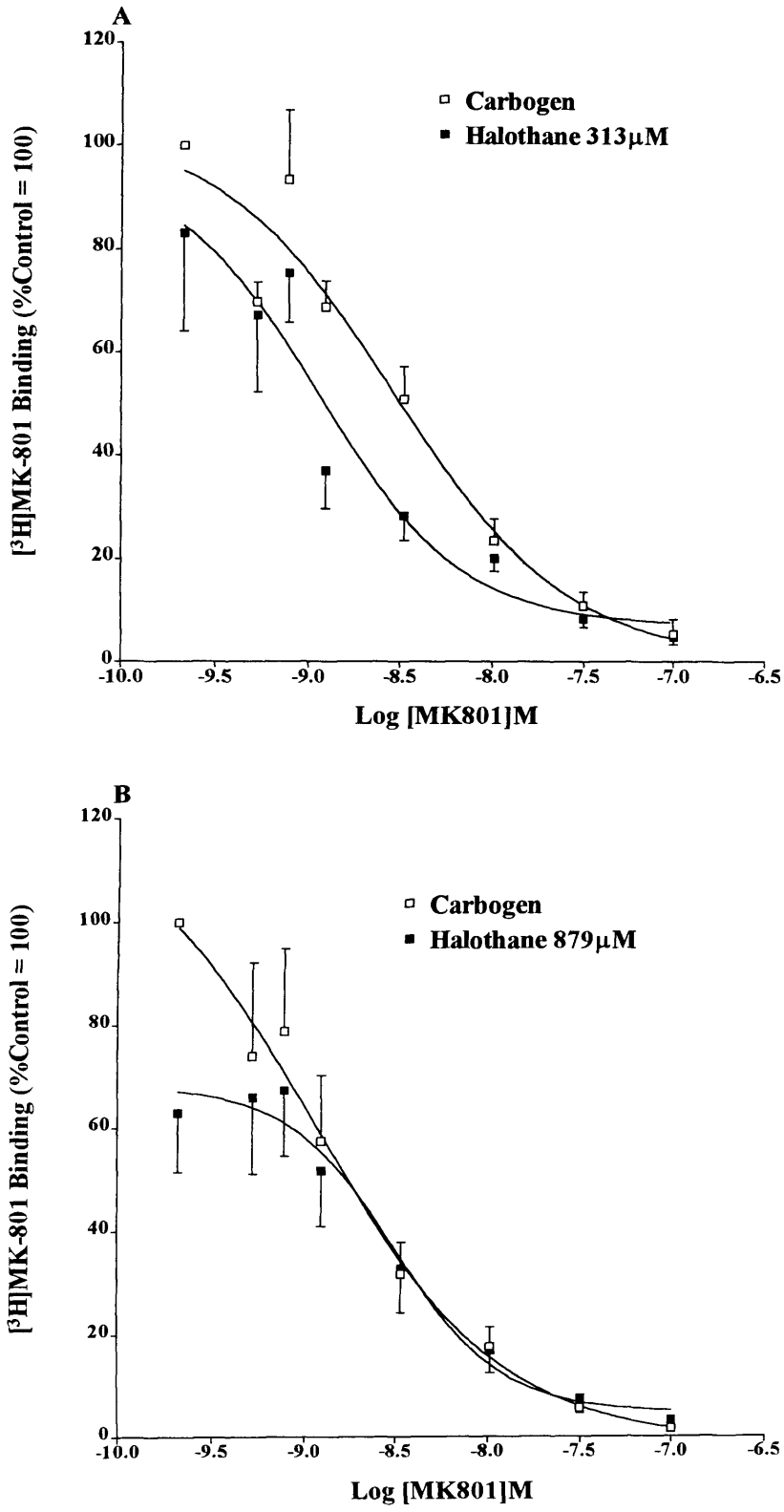


Fig 7.8. Halothane at 313.22±27.37 and 879.18±35.11μM failed to significantly affect the displacement of [^{3}H]MK-801 binding with MK-801. Data are mean±sem (n=4).

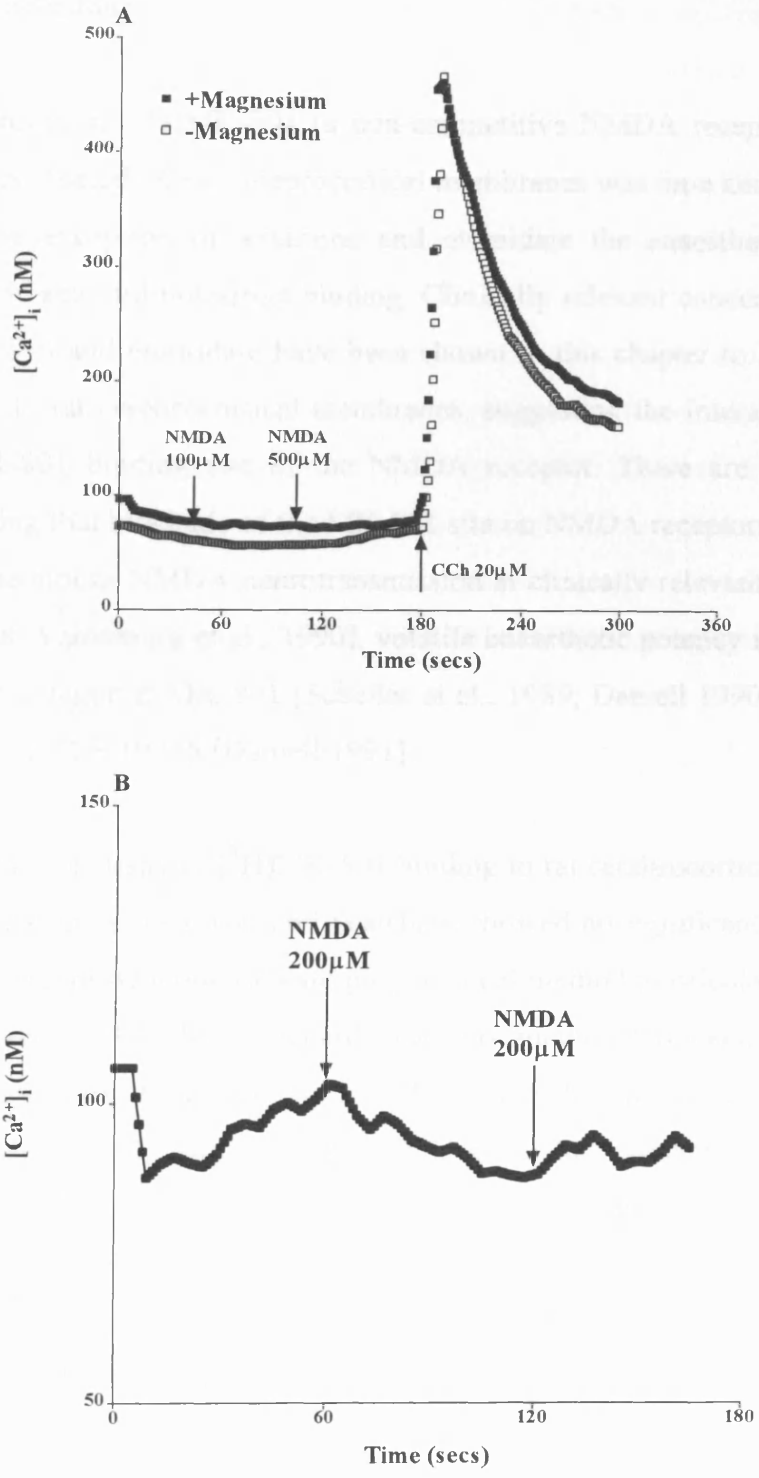


Fig 7.9. NMDA application did not increase $[Ca^{2+}]_i$ in neither SHSY-5Y human neuroblastoma (passage number 72) (A) nor undifferentiated P19 murine carcinoma (passage number 8) (B) cell lines. A & B are representative experiments of 3 and 6 respectively.

7.4. Conclusions

The binding of [^{3}H]MK-801 (a non-competitive NMDA receptor antagonist) to NMDA receptors located on rat cerebrocortical membranes was time and concentration dependent. With the exception of ketamine and etomidate the anaesthetic agents, morphine and barbituric acid did not affect binding. Clinically relevant concentrations of ketamine (*rac*, *R*- and *S*+) and etomidate have been shown in this chapter to interact with [^{3}H]MK-801 binding to rat cerebrocortical membranes, suggesting the interaction of these agents with the MK-801 binding site of the NMDA receptor. There are several lines of evidence suggesting that blockade of the MK-801 site on NMDA receptors has an anaesthetic effect: ketamine blocks NMDA neurotransmission at clinically relevant concentrations [Davies et al., 1988; Yamamura et al., 1990], volatile anaesthetic potency is increased by the NMDA receptor antagonist MK-801 [Scheller et al., 1989; Daniell 1990] and the NMDA receptor antagonist CGS 19755 [Daniell 1991].

The saturation assay of [^{3}H]MK-801 binding to rat cerebrocortical membranes analysed by rectangular hyperbola, Hill and Scatchard showed no significant differences in the B_{max} or the K_d. The introduction of a simple graphical method to calculate binding parameters was introduced in 1949 by Scatchard, and this method has been widely used by receptor pharmacologists. However, Scatchard analysis has received some criticism [Burgisser 1984; Klotz 1982; Munson 1983]. The problems associated with Scatchard include a clustering of data points on the x-axis, a scattering of data points following transformation, the saturation of the radioligand is difficult to estimate, no account for background radioactivity, improper subtraction of NSB and the occurrence of errors during separation of bound and free radioligand. In this study there were no significant differences in B_{max} or K_d using the other methods of analysis, therefore it is appropriate to use the Scatchard analysis of saturation binding isotherm data provided saturation has been reached. The estimated B_{max} and K_d values achieved in this study, when analysed by Hill also produced a mean slope factor of 1.16±0.1 implying that the binding of [^{3}H]MK-801 is to a single population of receptors.

Pichiule et al., 1996 reported B_{max} values in the cerebral cortex, hippocampus and corpus striatum of approximately 2905, 3794 and 2387fmol/mg protein respectively and Burke et al., 1995 reported a B_{max} in cortical/hippocampal membranes of approximately 4000fmol/mg protein. The B_{max} values achieved by Pichiule et al., and Burke et al., differed from the B_{max} estimated in this study. The reason for these differences is unclear but may be due to the age of the rats used, as cerebrocortical membranes used in our study are from 6-10 week old rats and the rats used by Pichiule and colleagues are 3 weeks old. The difference between the value in this study and that reported by Burke et al., may be due to the mixture of cortex and hippocampus in the preparation of the membranes.

NMDA receptors are ligand gated ion channels with modulatory sites for glycine in addition to the glutamate/NMDA sites. The removal of glycine alone from the binding assay in this study did not affect the binding, but removal of glutamate alone and both glutamate and glycine significantly reduced the binding by 16.02±1.77% and 46.76±2.9% respectively. Although in this thesis glycine appears to act as a positive modulator of [^{3}H]MK-801 binding, Kaplita and Ferkany 1990 have shown that glycine partially inhibits the specific binding of 7.5nM [^{3}H]CGS (a competitive NMDA antagonist) with an IC_{50} of 179±69nM. In contrast, glutamate has been reported to dose-dependently increase, and glycine was found to augment (more apparent in the presence of <10μM glutamate), the binding of 1nM [^{3}H]MK-801 to rat cerebrocortical membranes after a 1hour incubation at 37°C [Martin et al., 1995]. As the concentration of glutamate used in this chapter was 100μM, the removal of glycine would be unikely to affect binding. If the glutamate concentration was decreased perhaps a significant augmentation would have been observed.

At the NMDA receptors there is also a site to which dissociative anaesthetic agents act as non-competitive antagonists. The isomers and racemic mixture of ketamine displaced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes. Following Hill analysis, the slope factors for MK-801 and ketamine (rac, R- and S+) were approximately 1, indicating that the binding was to a single population of binding sites. When allowing for the binding to plasma proteins the free serum concentration of ketamine is 10-16μM [Frenkel et al.,

1993; Reilly 1994]. As the estimated K_i values are in the range of 0.57-2.7μM, the displacement produced occurs at a clinically relevant concentration.

Neither barbituric acid, the anaesthetic agents propofol, thiopentone and pentobarbitone nor the analgesic agent morphine at clinically relevant concentrations displaced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes. This would suggest that the mechanism of the inhibitory effects of propofol, thiopentone, pentobarbitone and morphine on 46mM K^+-evoked glutamate release from rat cerebrocortical slices is not at the MK-801 site on the NMDA receptor. The anaesthetic agent etomidate displaced the binding of [^{3}H]MK-801 to rat cerebrocortical membranes with an estimated $pK_i(K_i)$ of 5.4±0.02(4.01μM). When subtracting the percentage bound to plasma proteins, the free serum concentration of etomidate is ~2.5μM [Frenkel et al., 1993; Reilly 1994].

Halothane at concentrations of 313.22±27.37 and 879.18±35.11μM appeared to either decrease or mask the maximum number of binding sites, and to decrease the K_i for the displacement of [^{3}H]MK-801 binding with MK-801, compared to the paired control values in the presence of carbogen. However these effects were small and failed to reach statistical significance. As halothane appeared to decrease the maximum number of binding sites, it might imply that halothane was acting as a non-competitive antagonist at the MK-801 site located on the NMDA receptor complex. A decrease in the glutamate stimulated binding of [^{3}H]MK-801 was reported in the presence of clinically relevant concentrations of halothane, enflurane, methoxyflurane, chloroform and diethyl ether, reversible upon the removal of the anaesthetic agent [Martin et al., 1995]. Inclusion of glycine in the incubation medium attenuated the anaesthetic-induced inhibition of glutamate-sensitive [^{3}H]MK-801 binding, confirming a modulatory role for glycine [Martin et al., 1995].

Undifferentiated SH-SY5Y human neuroblastoma and P19 cell lines did not produce an increase in $[Ca^{2+}]_i$ suggesting that NMDA receptors were unlikely to be present. If NMDA receptors were present in SH-SY5Y cells, the removal of Mg^{2+} from the buffer should have relieved the voltage dependent block and produced an increase in $[Ca^{2+}]_i$ in response to NMDA application however no increase was observed. The application of 20μM CCh

to SH-SY5Y cells increased the $[Ca^{2+}]_i$ from 100nM to ~500nM in the absence and presence of Mg^{2+}, illustrating the activation of muscarinic receptors leading to the opening of Ca^{2+} channels production of IP_3 and release of Ca^{2+} from intracellular stores. Saturation analysis with ~25nM [^{3}H]MK-801 showed little differences in the total and NSB binding of either cell line. Retinoic acid-treated murine P19 embyonal carcinoma cells has been reported to induce neuronal differentiation [Edwards and McBurney 1983]. Using electrophysiological and molecular biology techniques, differentiated P19 cells have been reported to express NMDA and kainate receptors and are susceptible to glutamate neurotoxicity [Ray and Gottlieb 1993; Turetsky et al., 1993]. In this study no evidence was found for NMDA expression. However it should be noted that the full characterisation of Ca^{2+} responses in P19 cells could not be accomplished as the cells lysed during de-esterification. Further studies using adherent differenciated cells are indicated.

Chapter 8. General Discussion and Conclusions

Glutamate is the principal excitatory amino acid neurotransmitter within the CNS. This neurotransmitter has been implicated in the mechanism of anaesthesia [Puil and El-Beheiry 1990] and in neurodegenerative conditions [Nicholls and Attwell 1990]. Both intravenous and inhalation anaesthetic agents have been reported to affect glutamatergic transmission inhibiting its excitatory actions [Bickler et al., 1995]. An enhancement of synaptic glutamate concentration has been associated with head injuries, ischaemia and neurodegenerative diseases [Nicholls and Attwell 1990]. Several animal studies have reported that neurodegenerative disorders occur either as a consequence of excessive release of glutamate [Inglis et al., 1990; Bullock et al., 1991A&B; Kawamata et al., 1991] or inhibition of the uptake transporters resulting in excitotoxic damage to neurons and glia via overactivation of pre- and postsynaptic glutamate receptors [Rothstein et al., 1993]. Any effect of anaesthetic agents on the release and/or uptake could not only provide further evidence to suggest that glutamatergic neurotransmission is involved in anaesthesia, but also interrupt exocytotic damage, possibly providing neuroprotection.

8.1. Summary of Results

The uptake of [^{3}H]L-glutamate into rat cerebrocortical and cerebellar synaptosomes was time-, concentration-, Na^+- and temperature-dependent and L-trans-PDC sensitive. The substitution of Na^+ with either choline or lithium significantly reduced the uptake in both cerebrocortical and cerebellar synaptosomes. At clinically relevant concentrations neither intravenous nor inhalation anaesthetic agents, nor morphine altered the uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes (Table 8.1).

Perfused rat cerebrocortical slices depolarised twice with 46mM K^+ produced monophasic releases of endogenous glutamate. The released glutamate was measured fluorimetrically based on the fluorescence properties of NADPH. Depolarised release of glutamate was Ca^{2+} mediated representing exocytotic release, as EGTA pre-treatment essentially abolished the release. Morphine dose-dependently inhibited evoked release of glutamate. DAMGO (μ-selective agonist) and spiradoline (κ-selective agonist) produced a cyprodime

(μ-selective antagonist) and nor-BNI (κ-selective antagonist) sensitive inhibition of evoked glutamate release respectively. ICI-199,441 (κ-selective agonist) significantly inhibited the evoked release of glutamate at high concentrations. DPDPE (δ-selective agonist) proved ineffective. DAMGO, DPDPE and spiradoline did not alter the basal release of glutamate. Nociceptin (ligand at the orphan receptor, ORL_1) produced a dose-dependent naloxone insensitive inhibition of evoked glutamate release (Table 8.1).

The intravenous anaesthetic agents ketamine, propofol and thiopentone at clinically relevant concentrations produced a dose-dependent inhibition of evoked release of glutamate. The inhalation anaesthetic agent halothane at clinically relevant concentrations also dose-dependently inhibited release. At single doses, MK-801 (non-competitive antagonist at the NMDA receptor), isolfurane, pentobarbital but not barbituric acid significantly inhibited depolarised release (Table 8.1).

The binding of [^{3}H]MK-801 to a single population of NMDA receptors on rat cerebrocortical membranes was time and concentration dependent. The racemic mixture and S+ and R- isomers of ketamine and the intravenous anaesthetic agent etomidate significantly displaced the binding of [^{3}H]MK-801 at clinically relevant concentrations. Barbiturates (barbituric acid, thiopentone and pentobarbital), propofol, morphine and halothane failed to displace the binding of [^{3}H]MK-801 (Table 8.1).

Table 8.1. Summary of the effects of intravenous and inhalation anaesthetic agents, opioid agents and nociceptin on the uptake of [^{3}H]L-glutamate, the release of endogenous glutamate and the binding of [^{3}H]MK-801 to rat cerebrocortical membranes.

Agent	Uptake (synaptosomes)				Release		Binding
	Cerebrocortical		Cerebellar		Cerebrocortical Slices		Cerebrocortical Membranes
	Basal	46mM K^+	Basal	46mM K^+	Basal	46mM K^+	[^{3}H]MK-801/MK-801 displacement of [^{3}H]MK-801
Propofol*	↓(13,22,35%)[a]	↓(65%)[b]	↓(54,63%)[c]	↓(71%)[b]	ns	↓(IC_{50}=20.7μM)	↔
Ketamine (rac), (S+), (R-)	↔, ns, ns	↔, ns, ns	↔, ns, ns	↔, ns, ns	ns, ns, ns	↓(IC_{50}=18.2μM), ns, ns	d(K_i=638nM), d(K_i=568nM), d(K_i=2703nM)
Thiopentone*	↔	↓(40%)[b]	↔	↓(30%)[b]	ns	↓(IC_{50}=10.9μM)	↔
Pentobarbital	ns	ns	ns	ns	ns	↓(100μM)	↔
Etomidate	ns	ns	ns	ns	ns	ns	d(K_i=4μM)
Halothane	↔	↔	↔	↔	ns	↓(IC_{50}=132μM)	↔
Isoflurane	↔	↔	↔	↔	ns	↓(435μM)	ns
Morphine	↔	↔	↔	↔	ns	↓(IC_{50}=71nM)	↔
DAMGO	ns	ns	ns	ns	↔	↓(1μM)	ns
DPDPE	ns	ns	ns	ns	↔	↔	ns
Spiradoline	ns	ns	ns	ns	↔	↓(1 and 100μM)	ns
Nociceptin	ns	ns	ns	ns	ns	↓(IC_{50}=51nM)	ns

Key: ↓, inhibited; ↔, no change; ns, not studied; d, displaced. * [a] Inhibition at 30, 100 and 300μM respectively, [b] inhibition at 300μM, [c] inhibition at 100 and 300μM respectively.

8.2. [^{3}H]L-Glutamate Uptake

The K_m for the uptake of [^{3}H]L-glutamate into cerebrocortex was 8.61±1.91μM and was close to the original EAAC1 uptake transporter cloned by Kanai and Hediger. As the K_m value for the cerebellum was significantly different from the cerebrocortex, 2.23±0.33μM, it is possible that a different transporter is responsible. In the cerebellum the K_m was close to that reported for GLT-1 and EAAT4. Pines and colleagues located GLT-1 on glia, and as our preparation of synaptosomes is unlikely to possess glia, the transporter is unlikely to be GLT-1. As EAAT4 has been located in human cerebellum [Fairman et al.,1995], the transporter expressed in the cerebellum may be the rat equivalent of the EAAT4 transporter.

Propofol was the only intravenous anaesthetic agent to produce a significant inhibition in the basal uptake of [^{3}H]L-glutamate into cerebrocortical and cerebellar synaptosomes. The inhibitions were small and achieved using concentrations at the top of the clinically relevant range. The addition of 46mM K^+ to synaptosomes increased the inhibition achieved by propofol, and also produced a significant inhibition in the presence of thiopentone. A greater inhibition in the uptake of [^{3}H]L-glutamate was achieved in the presence of 46mM K^+, and may result in K^+ or drug modulated pump reversal. The clinical relevance of the effects of propofol on uptake and release will be discussed later.

8.3. Endogenous Glutamate Release

In this thesis, perfused rat cerebrocortical slices were used to study the release of endogenous glutamate. Fractions collected were analysed for glutamate using an adapted fluorimetric method of Nicholls and Sihra (1986). The addition of 46mM K^+ to perfused rat cerebrocortical slices evoked mononphasic releases of glutamate, S_1 and S_2. The amount of cerebrocortical slices from one rat cerebrocortex was sufficient to perform 3 experiments (1 control and 2 drug treated). One low and one high concentration of the agent under investigation was tested per cerebrocortex, ensuring a fair comparison between perfusion runs. Between experiments (from the same rat and different rats) glutamate release (basal and evoked) differed, therefore the results were expressed either relative to the mean of the first three basal samples or as an S_2/S_1 ratio. 46mM K^+-depolarisation of perfused rat cerebrocortical slices produced a regulated exocytotic release of glutamate as

the removal of Ca^{2+} from the perfusion buffer and the addition of EGTA essentially abolished evoked release. Glutamate is one of many neurotransmitters released in a Ca^{2+} dependent fashion (Table 8.2).

Table 8.2. Classification of the main neurotransmitters released via Ca^{2+}-mediated exocytosis into the CNS and their presumed effect on neurons [[1]Iredale et al., 1993; [5&6]Jeftinja et al., 1996; [1-8]McMahon and Nicholls 1991].

Neurotransmitter	Effect on Neurons
Noradrenaline[1]	Mainly Inhibitory (depending on the subtype)
Dopamine[2]	Inhibitory and Excitatory (depending on the subtype)
5-HT[3]	Inhibitory and Excitatory (depending on the subtype)
Acetylcholine[4]	Excitatory
Glutamate[5]	Excitatory
Aspartate[6]	Excitatory
GABA[7]	Inhibitory
Glycine[8]	Inhibitory (Augments glutamates actions at the NMDA receptor)

8.4. Opioid Interaction with Glutamatergic Neurotransmission

Opioid receptors couple to pertussis toxin-sensitive G-proteins (Gi/Go), and as such negatively couple to adenylyl cyclase reducing cAMP, close VSCCs and activate an outward K^+ current, hyperpolarising the membrane thereby reducing neuronal excitability [Lambert 1995]. In addition to their negative effects on neuronal excitability and neurotransmitter release, they also have stimulatory effects, activating phospholipase C producing IP_3 and DAG [Smart and Lambert 1996]. We have not shown any direct stimulatory effects of opioids on basal glutamate release as DAMGO, DPDPE and spiradoline proved ineffective. However μ-, δ- and κ-opioids have been reported to exert stimulatory effects on neurotransmission [Crain and Shen 1990], increasing the rate of

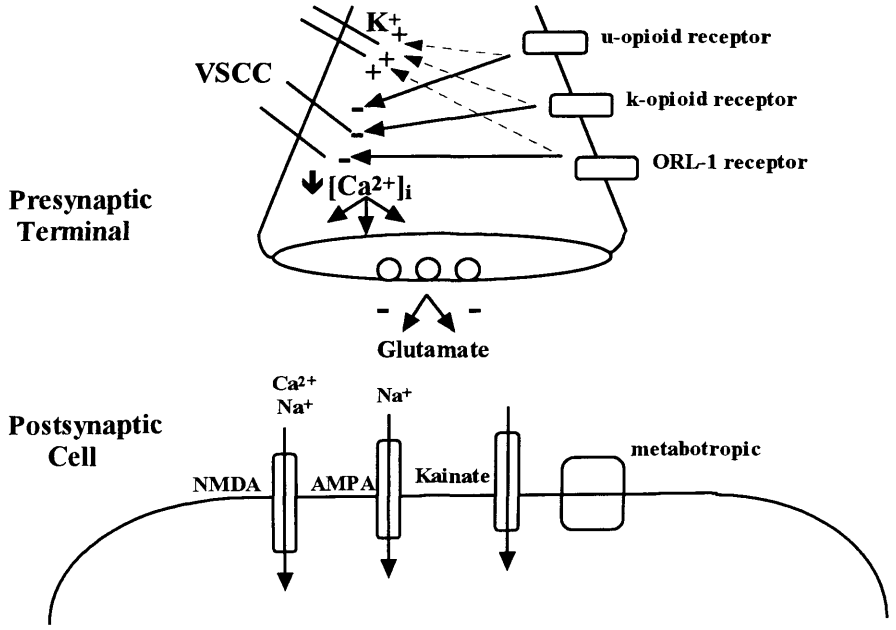
neuronal firing [Lin and Carpenter 1994] and prolongation of the action potential [Crain and Shen 1990; Shen and Crain 1994].

The inhibitory effect of opioid agonists on glutamate release previously reported is also confirmed in this thesis [Bradford et al., 1986; Ueda et al., 1995; Hill and Brotchie 1995; Hayward et al., 1995]. Morphine dose-dependently inhibited the release of 46mM K^+-evoked glutamate from perfused rat cerebrocortical slices. Further analysis of the dose-dependent inhibition yielded an estimated IC_{50} of 71nM. From the data in Chapter 3, morphine did not affect the uptake of [^{3}H]L-glutamate into either cerebrocortical or cerebellar synaptosomes. Therefore the inhibitory effect seen in Chapter 5 on 46mM K^+-evoked glutamate release from rat cerebrocortical slices is likely to directly involve the release process (i.e. closing VSCC and hyperpolarising). DAMGO and spiradoline also produced an inhibition of evoked glutamate release reversed by cyprodime and nor-BNI respectively. The reversibility of these inhibitions by the selective antagonists illustrates an opioid receptor mediated effect. The inhibition achieved by spiradoline was at a high concentration, but as this inhibition was reversed by nor-BNI (a κ-selective antagonist), the inhibition was due to κ-receptor activation. Bradford et al., 1986 reported an inhibition by 100μM U50,488H (a κ-selective agonist) of veratrine stimulated release of glutamate in a WIN44441-3 sensitive manner from mammalian cerebral cortical slices. In contrast, Hill and Brotchie 1995 reported that the κ-agonist enadoline, produced a dose-dependent nor-BNI sensitive inhibition of 4-AP stimulated release of glutamate from rat and marmoset striatum, with a much lower concentration (IC_{50}s of ~8.7 and ~2.9μM respectively). Common to all the inhibitory effects of opioid agonists, the inhibition of evoked glutamate release was not complete, i.e. <100%. Ueda et al., 1995 reported a reduction in the capsaicin evoked glutamate release from rat spinal dorsal horn slices of ~85, ~77 and ~59% with DAMGO, morphine and DPDPE respectively. A maximum inhibition of 4-AP stimulated release of glutamate from rodent and primate striatum occurred at 300μM enadoline, 68 and 69% respectively [Hill and Brotchie 1995]. The nature of the opioid insensitive component is presently unknown.

8.5. Nociceptin Interaction with Glutamatergic Neurotransmission

A novel heptadecapeptide nociceptin (an endogenous ligand at the orphan receptor) has been shown in this thesis to produce an inhibition of evoked glutamate release. The inhibition achieved was naloxone insensitive, illustrating a non-opioid receptor mediated event. At the present time no other studies have examined the effect of this peptide on glutamatergic neurotransmission, and as the receptor for nociceptin (ORL-1) has ben located in areas of the limbic system [Mollereau et al., 1994] this might imply a role for nociceptin in learning and memory. For this statement to hold, a similar inhibition must be absent in at least the hippocampus. The results from Chapter 5 would indicate that as μ- and κ-opioid agonists and nociceptin specifically inhibit the 46mM K^+-evoked release of glutamate, there may be presynaptic opioid and ORL-1 (receptor for nociceptin) receptors located in the cortex controlling glutamate release.

Fig 8.1. Presynaptic μ- and κ-opioid and ORL_1 receptors inhibit glutamate release by decreasing Ca^{2+} and increasing K^+.



8.6. Anaesthetic Interaction with Glutamatergic Neurotransmission

To date the molecular basis of the action of general anaesthetic agents remains unknown. Three main sites of action have received much attention; excitatory transmission, ligand gated ion channels and VSCCs. A substantial study examining the effects of inhalation and

intravenous anaesthetic agents on 46mM K^+-evoked glutamate release from rat cerebrocortical slices is reported in Chapter 6. The aim was to explore the hypothesis that glutamatergic neurotransmission is involved in anaesthesia.

Intravenous anaesthetic agents, ketamine, propofol and thiopentone inhibited 46mM K^+-evoked release of glutamate from rat cerebrocortical slices with IC_{50} values of 18.2, 20.7 and 10.9μM respectively. The free serum concentration of propofol following TIVA is around 1μM. The concentration of propofol following infusion in whole rat brain was reported by Shyr et al., 1995 to be 8.5 times that in plasma. Propofol at 300, 100 and 30μM produced significant inhibition of [^{3}H]L-glutamate uptake into rat cerebrocortical synaptosomes. If this effect were mimicked in cerebrocortical slices then the potency of propofol inhibition on evoked glutamate release would be underestimated. The question of relevance to the whole animal remains to be answered, and this thesis can only illustrate the effects of anaesthetic agents on glutamate release *ex vivo*. The degree of inhibition is important and perhaps lower than IC_{50} concentration should be included, see Section 8.8. However it is folly to compare the dose-response curve effect of an anaesthetic agent on the body (MAC), with the dose-response curve effect produced in a test-tube. As propofol reduces intracranial pressure, cerebral blood flow and the cerebral metabolic rate for oxygen [Ravussin and Strebel 1995] along with its inhibitory effects on glutamate release, it would appear that propofol would provide excellent anaesthesia.

Pentobarbitone and thiopentone but not barbituric acid decreased evoked glutamate release. The inhibition achieved may be due to the anaesthetic properties of thiopentone and pentobarbital, and not merely due to barbiturate structure as the non-anaesthetic barbiturate, barbituric acid was ineffective. In addition, this would also imply that the anaesthesia achieved by pentobarbitone and thiopentone, might involve glutamatergic neurotranmission.

Prior to the examination of the effect of the inhalation anaesthetic agents, halothane and isoflurane on evoked glutamate release, the aqueous concentration of these agents were measured by gas chromatography (GC), an important step often overlooked in many studies. Halothane dose-dependently inhibited evoked glutamate release with an IC_{50} of

132μM, isoflurane also inhibited at 435μM corresponding to ~0.44 and ~1.24 rat MAC respectively, clinically relevant concentrations. Halothane was reported by Schlame and Hemmings (1995) to inhibit the 4-AP- and veratridine-, but not the basal, K^+-, or ionomycin-evoked glutamate release and also to inhibit 4-AP- and K^+-evoked increase in free $[Ca^{2+}]_i$. Isoflurane, enflurane and halothane decreased K^+-evoked glutamate release, and K^+-evoked increase in $[Ca^{2+}]_i$ [Miao et al., 1995]. The decrease in K^+-evoked $[Ca^{2+}]_i$ was consistent with an inhibition of Ca^{2+} entry, possibly by specific voltage gated neuronal Ca^{2+} channels. Isoflurane and halothane have also been reported to depress glutamate mediated synaptic transmission [Maciver et al., 1996].

If a single common target site for anaesthesia existed, all anaesthetics would produce the same effect. Different pharmacological effects are observed depending on the anaesthetic used, thereby implying action at multiple molecular targets [Franks and Lieb 1993]. As the data in many reports [Brockmeyer and Kendig 1995; Bickler et al., 1995] (including this thesis) has illustrated an inhibitory effect of some anaesthetic agents on evoked glutamate release it is becoming increasing likely that one of the target sites for anaesthesia is excitatory neurotransmission. In agreement with this statement, Mantz et al., 1992 reported that riluzole, an agent that inhibits both the release and some postsynaptic effects of glutamate, was able to exert anaesthetic action alone, in addition to potentiating the anaesthetic effect of ketamine and thiopentone.

8.7. Anaesthetic Interaction with the MK-801 Site on the NMDA Receptor

Following depolarisation evoked glutamate release from presynaptic terminals, glutamate binds to its receptors located on the pre- and postynaptic terminal. Activation of the postsynaptic glutamate receptors directly may result in an increase in $[Ca^{2+}]_i$, activation of endonucleases, lipases, proteases and nitric oxide resulting in neuronal cell death. NMDA and metabotropic glutamate receptors have been reported to occur presynaptically [Conti et al., 1997; DeBiasi et al., 1996; Sanchez-Prieto et al., 1996], activation of which would increase $[Ca^{2+}]_i$ enhancing glutamate release and producing an overactivation of postsynaptic glutamate receptors. An inhibition of the presynaptic NMDA receptors would reduce the release of glutamate and the increase of $[Ca^{2+}]_i$.

MK-801 is a non-competitive antagonist of the NMDA receptor channel. Investigations into the effect of anaesthetic agents on this site on the NMDA receptor would provide clues as to the mechanism of the inhibitory effects of anaesthetic agents on glutamate release, i.e. block of presynaptic NMDA receptors would decrease release. However block of VSCC would also produce an inhibition.

The binding of [^{3}H]MK-801 to NMDA receptors on rat cerebrocortical membranes was displaced by ketamine as expected, as the site of binding for MK-801 is the same for ketamine. The increasing rank order of potencies for the displacement of [^{3}H]MK-801 binding was the R- isomer (K_i~2703nM), the racemic mixture (K_i~638nM) and the S+ isomer (K_i~568nM). The racemic mixture of ketamine is a mixture of equal proportions of the two isomers, however the clinical superiority of the S+ isomer has been described with respect to anaesthetic potency, extent of analgesia and effects during and after surgery [Hemplemann and Kuhn 1997]. The clinical superiority of the S+ isomer was reported to be due to its effect on NMDA receptors in central nervous tissue accompanied by opioidergic, noradrenergic, dopaminergic and serotoninergic mechanisms. Etomidate also displaced [^{3}H]MK-801 binding, yet this anaesthetic agent does not produce dissociative ketamine-like anaesthesia. Halothane at clinically relevant concentrations produced a small and non-significant reduction in the binding of [^{3}H]MK-801, and appeared to reduce the number of available binding sites for [^{3}H]MK-801. The use of MK-801 to block NMDA receptor activation alone and estimate the interaction of anaesthetic and analgesic agents is not sufficient. NMDA receptors are composed of a channel complex with numerous binding sites [Small and Buchan 1997], so for a full picture of the effects of agents on NMDA receptors, interactions with the glycine, glutamate/NMDA and polyamine sites must be performed.

8.8. Clinical Relevance of Anaesthetic/Analgesic Glutamatergic Inhibition

The data in this thesis has reported inhibitory effects of a range of anaesthetic/analgesic agents on evoked glutamate release from rat cerebrocortical slices *ex vivo*. Binding studies also indicated that the inhibition produced by some agents may involve an interaction with the glutamate NMDA receptor. Franks and Lieb 1994 reported that although general anaesthetic agents at high enough concentrations can act non-specifically on a wide variety

of neuronal sites, at clinical concentrations they are more selective. They may exert their primary effects at a relatively small number of CNS targets, i.e. postsynaptic ligand gated ion channels, presynaptic autoreceptors or VSCC. In summary the data presented in this thesis suggests that glutamatergic transmission is important for anaesthetic action and underscores the need for further studies.

Chapter 9. References

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Publications Arrising from this Thesis

Full Papers

1. Nicol B, Rowbotham DJ and Lambert DG. Glutamate uptake is not a major target site for anaesthetic agents. British Journal of Anaesthesia, 1995; 75: 61-65.

2. Nicol B, Rowbotham DJ and Lambert DG. μ- and κ-opioids inhibit K^+ evoked glutamate release from rat cerebrocortical slices. Neuroscience Letters, 1996; 218: 79-82.

3. Nicol B, Rowbotham DJ, Lambert DG, Smart D and McKnight AT. Nociceptin inhibits K^+ evoked glutamate release from rat cerebrocortical slices. British Journal of Pharmacology (special report), 1996; 119: 1081-1083.

4. Nicol B, Rowbotham DJ and Lambert DG. Volatile and intravenous anaesthetics decrease K^+-evoked glutamate release from rat cerebrocortical slices. Journal of Neurochemistry (in preparation).

5. Nicol B, Rowbotham DJ and Lambert DG. Interaction of anaesthetic agents with the MK-801 site at the glutamate NMDA receptor. British Journal of Pharmacology (in preparation).

Abstracts (O = oral communication , P = poster)

6. Nicol B, Rowbotham DJ and Lambert DG. Comparison of the uptake of [^{3}H]glutamate into rat cerebrocortical and cerebellar synaptosomes. Effects of anaesthetic agents. Biochemical Society Transactions, 1994; 22: 410S (P).

7. Nicol B, Rowbotham DJ and Lambert DG. Morphine inhibits glutamate release from rat cerebrocortical slices. British Journal of Pharmacology, 1996; 117:293P (P).

8. Nicol B, Rowbotham DJ and Lambert DG. Is glutamate release from rat cerebrocortical slices opioid receptor subtype selective? British Journal of Anaesthesia, 1996; 77: 282P (O).

9. Nicol B, Rowbotham DJ and Lambert DG. Effects of NMDA receptor antagonists and thiopentone on glutamate release from rat cerebrocortical slices. British Journal of Anaesthesia, 1997; 78; 449-450P (O).

10. Nicol B, Rowbotham DJ and Lambert DG. Halothane decreases K^+-evoked glutamate release from rat cerebrcortical slices. British Journal of Anaesthesia, 1997; 79: 140P (P).

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