CHARACTERISATION OF THE BINDING OF TWO NOVEL GLYCINE SITE ANTAGONISTS TO CLONED NMDA RECEPTORS

BELA CHOPRA
SUBMISSION FOR A PhD DEGREE

THE SCHOOL OF PHARMACY, UNIVERSITY OF LONDON
Dedicated to my Dad, Mum
& to the loving memory of Cha Ji & Manju
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ABSTRACT

The focus of this thesis was to investigate the N-methyl-d-aspartate (NMDA) receptor subtype-selectivity of 3-[2-(phenylaminocarbonyl)ethyl]-4,6-dicloroindole-2-carboxylic acid sodium salt (GV150,526A) and E-4,6-dichloro-3-(2-oxo-1phenyl-pyrrolidin-3-ylidenemethyl)-1H-indole-2-carboxylic acid (GV196,771A) by assaying their ability to inhibit the radioligand binding of [³H](E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid (MDL105,519), to human embryonic kidney (HEK) 293 cells transfected with various combinations of NMDA receptor clones.

Competition binding between [³H] MDL105,519 and GV150,526A to both NR1-1a and NR1-2a subunits was best fit to a one-site model and there were no significant difference in affinity of GV150,526A for the different splice forms. In contrast, GV196,771A binding to each of the NR1 splice variants were best fit by a two-site model with the same affinity for both NR1-1a and NR1-2a splice forms. Interestingly, the competition profiles of both GV150,526A and GV196,771A to heteromeric NR1-1a/NR2s were best fit to a two-site compared to a one-site model. A small but significant selectivity was observed for both GV150,526A and GV196,771A where the rank order of decreasing affinity for GV150,526A was NR1-1a/NR2D > NR1-1a/NR2A = NR1-1a/NR2B > NR1-1a/NR2C. In contrast to GV150,526A, GV196,771A displayed a four-fold lower affinity for NR1-1a/NR2A compared to NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors.

The biphasic competition curves of [³H] MDL105,519 radioligand binding to heteromeric NMDA receptors by both GV150,526A and GV196,771A were investigated by various approaches including studying the competition profiles for the inhibition [³H] MDL105,519 radioligand binding to NR1-1a/NR2A receptors by a series of glycine site ligands with diverse chemical structures. Of the five compounds investigated L689,560 exhibited similar behaviour to GV150,526A with respect to inhibition binding of [³H] MDL105,519 to single
NR1-1a subunits and NR1-1a/NR2A receptors. Secondly, intact cell surface radioligand binding was performed to investigate the displacement by both GV150,526A and GV196,771A of \( [^3H] \) MDL105,519 radioligand binding to cell surface NR1-1a/NR2A receptors versus total receptor populations i.e. NR1-1a and NR1-1a/NR2A. However, both the intact cell surface radioligand binding and control experiments indicated that the NR1-1a subunit alone may be expressed at the cell surface. Furthermore, allosteric modulation by glutamate on the inhibition binding of \( [^3H] \) MDL105,519 by GV150,526A for native membranes under non-equilibrium conditions suggested that the biphasic competition curves may be due to allosteric interactions within a receptor complex.

On the basis of these results presented in this thesis it is proposed that glycine site antagonists may be subdivided into two pharmacological classes, i.e. those that show simple competitive behaviour with regard to inhibition of \( [^3H] \) MDL105,519 radioligand binding and those that result in complex inhibition curves.
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<th>Definition</th>
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<tr>
<td>ACPD</td>
<td>1-aminocyclopentane-1,3-dicarboxylate</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>AP5</td>
<td>D-2-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>AP7</td>
<td>D-2-amino-7-phosphonoheptanoic acid</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPA</td>
<td>(RS)-2-amino-3(3-hydroxy-5-tert-butylisoxal4-yl)propanoic acid</td>
</tr>
<tr>
<td>Bmax</td>
<td>Maximum number of receptors per mg protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CaMKII</td>
<td>Calmodulin-dependent kinase II</td>
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<tr>
<td>cAMP</td>
<td>Cyclic 3',5'-adenosine monophosphate</td>
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<td>CCG</td>
<td>2S,3R,4S derivative of 2-(carboxycyclopropyl) glycine</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>cGMP</td>
<td>Cyclic guanidino monophosphate</td>
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<td>CGP 39653</td>
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<td>((+-)trans-4-[2-(4-azidophenyl)acetylamino]-5,7-dichloro-1,2,3,4 tetrahydro-quinoline-2-carboxylic acid)</td>
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<tr>
<td>CGS 19755</td>
<td>Cis-4-(phosphonomethyl)piperidine-2-carboxylic acid</td>
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<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>7-CLKA</td>
<td>7-chlorokynurenic acid</td>
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<td>CMV</td>
<td>Cytomegalovirus</td>
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<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
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<td>Central nervous system</td>
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<td>Ethylenebis(oxyethylenenitrilo)tetracetic acid</td>
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<td>ELISA</td>
<td>Enzyme linked immunoadsorbent assay</td>
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<td>Foetal calf serum</td>
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<td>Guanine nucleotide-binding regulatory protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
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<td>GluR</td>
<td>Glutamate receptor subunit</td>
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<td>G-protein coupled receptors</td>
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<td>HBS</td>
<td>HEPES buffered saline</td>
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<td>HBSS</td>
<td>Hanks buffered salt solution</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
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<td>Horseradish peroxidase</td>
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<td>L701,324</td>
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<td>LIVBP</td>
<td>Leucine/isoleucine/valine-binding protein</td>
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<td>Definition</td>
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<tr>
<td>LOABP</td>
<td>Lysine/arginine/ornithine-binding protein</td>
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<td>2-amino-2-(2-carboxycyclopropan-1-yl)-3-(dibenzopyran-4-yl)propanoic acid</td>
</tr>
<tr>
<td>LY354740</td>
<td>(+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylate</td>
</tr>
<tr>
<td>M</td>
<td>Membrane region</td>
</tr>
<tr>
<td>MAP-4</td>
<td>(S)-2-amino-2-methyl-4-phosphonobutanoic acid/alpha-methyl</td>
</tr>
<tr>
<td>MCAo</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MCPG</td>
<td>α-methyl-4-carboxyphenylglycine</td>
</tr>
<tr>
<td>MDL105,519</td>
<td>(E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor subunit</td>
</tr>
<tr>
<td>MK801</td>
<td>(+)-5-Methyl-10,11-dihyro-5H-dibenzo[a,d]cyclohepten-5,10-imine</td>
</tr>
<tr>
<td>MPPG</td>
<td>(R,S)-α-methyl-4-phosphonophenylglycine</td>
</tr>
<tr>
<td>MPTG</td>
<td>(R,S)-α-methyl-4-tetrazolylphenylglycine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular mass</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSOP</td>
<td>(R,S)-alpha-methylserine-O-phosphate</td>
</tr>
<tr>
<td>n_h</td>
<td>Hill coefficients</td>
</tr>
<tr>
<td>NBQX</td>
<td>6-cyano-7-sulphamoylbenzo[f]quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NR</td>
<td>NMDA receptor subunit</td>
</tr>
<tr>
<td>NS-102</td>
<td>5-Nitro-6,7,8,9, tetrahydrobenzo[g]indole-2,3-dione-3-oxime</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBPs</td>
<td>Periplasmic binding proteins</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Phenycyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Phosphokinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Phosphokinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyl sulphonyl fluoride</td>
</tr>
<tr>
<td>PSD 95</td>
<td>Post-synaptic density 95</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD 95/ discs large/ zonula occuludentes</td>
</tr>
<tr>
<td>QBP</td>
<td>Glutamate binding protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAase</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>(RS)-PPG</td>
<td>(R,S)-4-phosphononophenylglycine</td>
</tr>
<tr>
<td>(S)-3C4HPG</td>
<td>(S)-3-carboxy-4-hydroxyphenylglycine</td>
</tr>
<tr>
<td>(S)-4C3HPG</td>
<td>(S)-4-carboxy-3-hydroxyphenylglycine</td>
</tr>
<tr>
<td>(S)-(4CPG)</td>
<td>(S) 4-carboxyphenylglycine</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SYM2081</td>
<td>(2S,4R)-4-methylglutamic acid</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-HCl, EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>NNNN'-Tetramethylenediamine</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>TGE</strong></td>
<td>Tris-HCl, glucose and EDTA buffer</td>
</tr>
<tr>
<td><strong>TRIS</strong></td>
<td>Tris(hydroxymethyl)methylamine</td>
</tr>
<tr>
<td><strong>U.V.</strong></td>
<td>Ultra-violet</td>
</tr>
<tr>
<td><strong>Z-CBQA</strong></td>
<td>(Z)-amino-3-[2'-(3',5'-dioxo-1',2',4'-oxadiazolidinyl)]cyclobutane-1-carboxylic acid</td>
</tr>
</tbody>
</table>
FOREWORD

As the 21st century begins, the neuroscientist is once again faced with a combination of hope and excitement. One can look back over the last hundred years with incredulity as our knowledge has advanced even further than could have been conceived at the beginning of the century. The constant exchange of imagination and inspiration has demanded a novel and a comprehensive way to look at challenging problems. We are now faced with vaster opportunities and horizons for the new century.

"The flood of new ideas and experiments in the late 1970s and 1980s - recombinant DNA techniques, site-directed mutagenesis, highly mechanised DNA sequencing, spectacular advances in genetic engineering and vivid new methods for visualising complex molecular structures - all of these advances demanded a fresh coherent presentation of current biochemical science. The new complexities revealed by recent experimental data, have paradoxically, made the science easier to comprehend. The new data have uncovered new organising principles. New methods of presentation have made it possible to dramatically visualise the three dimensional structure of complex macromolecules by artistic rendition and advanced computer graphics." - Irving Geis (1989).
CHAPTER 1

INTRODUCTION
CHAPTER ONE: INTRODUCTION

1.1 L-GLUTAMATE AS A NEUROTRANSMITTER

L-Glutamate is the principal excitatory neurotransmitter in the brain. Curtis and his colleagues in the late 1950s demonstrated that the acidic amino acids, L-glutamate and L-aspartate, were able to excite all types of central neurons (Curtis et al., 1959). Since then it has been demonstrated that L-glutamate fulfils all the criteria used to define a neurotransmitter including its abundance in the adult central nervous system (CNS) and synthesis within the brain by different biochemical pathways. For example, glutamate can be synthesised from 2-oxoglutarate, an intermediate in the Krebs cycle, by transamination or via the enzyme glutamic acid dehydrogenase. L-Glutamate can also be produced from glutamine which is normally abundant in the interstitial space and is deaminated in neurons by the enzyme, glutaminase to form glutamate. Further evidence in support of the role of L-glutamate as a neurotransmitter is its pre-synaptic localisation in specific neurons and its release following physiological stimulation in a Ca²⁺-dependent manner. L-Glutamate mediates many important actions in the CNS including its involvement in memory formation, synaptic plasticity (section 1.15) (Bliss and Collingridge, 1993) and the pathophysiology of many neurological disorders such as ischaemic or hypoxic damage (section 1.15) (reviewed in Meldrum and Garthwaite, 1990).
1.2 THE DIVERSITY OF GLUTAMATE RECEPTORS

The diverse roles of L-glutamate in the CNS as mentioned above, are mediated through multiple excitatory amino acid receptors. Our current understanding of the glutamatergic field of receptors is due to the pioneering research of Watkins and co-workers who identified selective agonists and antagonists to characterise the glutamatergic-induced responses of neurons (Watkins et al., 1990). On the basis of this and other studies the general consensus is that there are three distinct glutamate receptor families. These receptor subtypes include the ligand-gated cation channels termed NMDA and non-NMDA receptors and those that modulate intracellular messengers via guanine nucleotide-binding regulatory (G)-proteins called the metabotropic glutamate receptors (mGluRs). The non-NMDA receptor family can be further subdivided into the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate receptors. Figure 1.0 shows the structures of the selective agonists of the glutamate receptor family.

The advent of molecular biology upheld this pharmacological classification of glutamate receptors and in addition, based only on their amino acid sequence similarity between glutamate receptor subunits identified a fourth group referred to as the orphan receptors (Yamazaki et al., 1992a; Lomeli et al., 1993). The phylogenetic tree of the ionotropic glutamate receptor subunits is shown in Figure 1.1.
1.3. NMDA RECEPTORS

The NMDA receptor takes its name from its selective agonist, NMDA, a synthetic analogue of glutamate (Watkins, 1962). This member of the ionotropic glutamate receptor family is a complex and intriguing macromolecule in that it requires the binding of both glutamate and the co-agonist, glycine, with the relief of a voltage-dependent Mg$^{2+}$ block for receptor activation (Mayer et al., 1984; Johnson and Ascher, 1987). The channel is primarily permeable to K$^+$, Na$^+$ and Ca$^{2+}$ ions (Mayer and Westbrook, 1987). Several distinct ligand binding sites other than those for glutamate and glycine have been identified on the NMDA receptor that allow modulation of the primary response of the receptor including the channel pore, divalent cation and polyamine sites. Figure 1.2 shows a schematic representation of the NMDA receptor along with its modulatory binding sites.

1.4 PHARMACOLOGICAL PROPERTIES OF NMDA RECEPTORS

1.4.1 NMDA/glutamate binding site

As stated above, the NMDA receptor was distinguished from the other ionotropic glutamate receptors following the synthesis of its selective agonist, NMDA (Watkins, 1962). The agonists of the glutamate binding site that succeeded NMDA include 2S,3R,4S derivative of 2-(carboxycyclopropyl) glycine (CCG) and (tetrazol-5-yl)
glycine. Both these compounds are more potent than NMDA (Shinozaki et al., 1989; Schoepp et al., 1991).

The research of Watkins and his colleagues focussed into the search for selective excitatory amino acid receptor antagonists to provide tools for understanding the physiological roles of the different types of ionotropic glutamate receptors (Watkins et al., 1990). This led to the development of NMDA receptor antagonists, D-2-amino-5-phosphonovaleric acid (AP5) and D-2-amino-7-phosphonoheptanoic acid (AP7) (Davis et al., 1981; Perkins et al., 1981). In subsequent years, cyclic derivatives of both AP5 and AP7 were described including, D-3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid (D-CPP-ene), cis-4-(phosphonomethyl)piperidine-2-carboxylic acid (CGS19755) (Selfotol) and (E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP37849). These ligands exhibited improved ligand affinity and selectivity compared with long chain phosphonate analogues i.e. AP5 and AP7 (Davis et al., 1986; Lehmann et al., 1988). Recently, an analogue of CGP37849 has been synthesised, D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid (CGP39653)(Sills et al., 1991). This antagonist proved to be an excellent tool for the study of the glutamate/NMDA binding site since its properties include high affinity and a slow dissociation rate thus allowing a filtration-based protocol to be employed in radioligand binding studies (see Appendix) (Sills et al., 1991).
1.4.2 Co-agonist glycine binding site

In the early 1980s, it was shown that [³H] glycine radioligand binding to membranes prepared from the adult rat cerebral cortex was insensitive to displacement by strychnine (Kishimoto et al., 1981). Furthermore, the distribution of [³H] glycine and [³H] strychnine binding in the adult rat forebrain was not co-localised but complementary (Bristow et al., 1986). These reports hinted towards the possibility that glycine binding sites distinct from the strychnine-sensitive glycine receptor may exist. In 1987, Johnson and Ascher, demonstrated that the magnitude of the electrophysiological responses of cultured neurons to applied NMDA were potentiated in the presence of sub-micromolar concentrations of glycine (Johnson and Ascher, 1987). This led to the revolutionary idea that the strychnine-insensitive glycine binding site is connected with the NMDA receptor. These findings were reinforced by autoradiographic studies where it was shown that NMDA-sensitive [³H] glutamate binding sites are co-localised with the strychnine-insensitive [³H] glycine binding sites in the rat brain (Bowery, 1987). Later, Kleckner and Dingledine, (1988) reported that glycine is an absolute requirement for the activation of NMDA receptors. Thus the term co-agonist was proposed for the glycine binding site on the NMDA receptor.

The dual role of glycine in activating both strychnine-sensitive and -insensitive binding sites directed research into the investigation of other possible selective endogenous ligands for the NMDA-coupled glycine binding site. Hashimimoto and colleagues, showed that D-serine can also act as an endogenous agonist for the glycine binding site.
of NMDA receptors (Hashimoto et al., 1993). Subsequently, other agonists acting at the strychnine-insensitive glycine binding site were identified, with a rank order of potency for native NMDA receptors being, glycine > D-serine > D-alanine > L-serine > L-alanine (Bonhaus et al., 1987; Reynolds et al., 1987; Snell et al., 1987; Kleckner and Dingledine, 1988).

In the last decade, there have been a number of glycine site antagonists developed many of which are derivatives of kynurenic acid for example, 5,7-dichlorokynurenic acid (5,7-DCKA) and 7-chlorokynurenic acid (7-CLKA). Glycine site antagonists that superseded these antagonists possess nanomolar potency for the glycine binding site and include (±)-4-trans-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline (L689,560) and MDL105,519 (reviewed in Leeson and Iverson, 1994; Dansyz and Parsons, 1998). Figure 1.3 shows the structure of these glycine site antagonists. However, these compounds have served as templates for the development of a new generation of glycine site antagonists including substituted indole-2-carboxylates. Both GV150,526A and GV196,771A belong to this class (Figure 1.3). GV150,526A displaces [³H] glycine binding to membranes prepared from adult rat cerebral cortex with an inhibition constant, Kᵢ = 3.2 nM (Mugnaini et al., 1997). In contrast, GV196,771A inhibits [³H] glycine to adult rat cerebral cortical membranes with a Kᵢ = 28 nM (Quartaroli et al., 1999). More recently, another compound emerged as a glycine-site antagonist, (+)-trans-4-[2-(4-azidophenyl) acetylamino]-5,7-dichloro-1,2,3,4-tetrahydro-quinolines-2-carboxylic acid ([³H] CGP61594) (Figure 1.3) (Honer et al., 1998). Interestingly, [³H] CGP61594 is also a photoaffinity labelled ligand and
can be used as a tool for the identification of structural elements of the glycine binding site as discussed in section 1.11 (Honer et al., 1998).

1.4.3 Ion channel binding site

In the early 1980s, it was shown that the dissociative anaesthetics, ketamine and phenycyclidine (PCP), selectively blocked the NMDA-induced excitation of rat and cat spinal neurons (Anis et al., 1983). These compounds were proposed to block NMDA-induced currents in a use- and voltage-dependent manner (Holney et al., 1985). Consequently, both PCP and ketamine are classified as non-competitive antagonists as they bind within the open channel pore of NMDA receptors (Monaghan and Cotman, 1985). Accordingly, the NMDA receptor must be liganded by both glutamate and glycine for the receptor to be in the correct configuration to allow the binding of these ligands (Loo et al., 1986). In subsequent years, a large number of open channel blockers have been developed derived from the PCP structure including, 1-(1-2-thienyl)-cyclohexyl-piperidine (TCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK801). The latter compound to date is the most potent and selective antagonist for the NMDA open channel binding site. This was demonstrated by both [³H] MK801 radioligand binding to rat brain membranes and electrophysiological studies on cultured neurons (Wong et al., 1986; Huettner and Bean, 1987).
1.4.4 Divalent cation binding sites

As mentioned above, the NMDA receptor channel differs in fundamental ways from other ionotropic glutamate receptors as the receptor channel is gated by both ligands and voltage. The NMDA receptor’s voltage-dependence follows directly from channel block by submillimolar concentrations of Mg\(^{2+}\) binding at a resting membrane potential of -80 mV (Nowak et al., 1984; Mayer et al., 1984). However, during membrane depolarisation the voltage-dependent block by Mg\(^{2+}\) is partially relieved thus allowing ionic influx through the activated NMDA receptor channel. Based on the measured voltage-dependence of block, Mg\(^{2+}\) has been proposed to bind to a site deep within the channel pore (Ascher and Nowak, 1988; Ruppersberg et al., 1994; Wollmuth et al., 1998). The molecular structural determinants of Mg\(^{2+}\) block are discussed in section 1.12.2.2.

In addition to the extracellular Mg\(^{2+}\) block, accumulating evidence points towards the existence of an intracellular Mg\(^{2+}\) ion binding site which can also exert a voltage-dependent block of NMDA receptor channels (Nowak et al., 1984; Johnson and Ascher, 1990). In contrast to the extracellular Mg\(^{2+}\) block, single channel recordings show that the intracellular Mg\(^{2+}\) block increases with depolarisation (Johnson and Ascher, 1990). Biophysical results demonstrated that both intracellular and extracellular Mg\(^{2+}\) block display considerably different dissociation rates (Johnson and Ascher, 1990) thus suggesting that both intracellular and extracellular Mg\(^{2+}\) block occurs at distinct binding sites within the pore of the NMDA receptor channel. Mutational evidence for disparate
localisation of these binding sites is shown in section 1.12.2.2.

Zinc ions can also inhibit NMDA receptor function in a voltage-independent manner indicating that Zn$^{2+}$ binds to a specific site distinct to the Mg$^{2+}$ binding site which is thought to be located on the extracellular surface of the NMDA receptor (Westbrook and Mayer, 1987; Reynolds and Miller, 1988).

1.4.5 Polyamine modulatory binding sites

The endogenous polyamines, spermidine and spermine, have multiple effects on NMDA receptor function. Ransom and Stec were the first group to show that these polycations enhance $[^3H]$ MK801 radioligand binding to NMDA receptors (Ransom and Stec, 1988). Following this finding, other modulatory actions of polyamines have now been characterised including potentiation of NMDA receptor currents in the presence of saturating concentrations of glycine and an increase in the affinity of NMDA receptors for glycine in an environment of low glycine concentration. The latter effect has been postulated to be due to a reduction of the rate of dissociation of glycine from the NMDA receptor (Benveniste and Mayer, 1993). Furthermore, extracellular spermine also causes a voltage-dependent channel block of NMDA receptors (Williams, 1997). Spermidine does not displace the binding of $[^3H]$ glycine or of the glutamate site agonist, $[^3H]$ CCP, thus suggesting a distinct polyamine modulatory site is present on the receptor (Ransom and Stec, 1988). The opposing actions of the polyamines mentioned above have been accounted for by there being two distinct polyamine binding sites on the NMDA
receptor (Rock and MacDonald, 1992).

1.4.6 Allosteric interactions between the glutamate and glycine binding sites on the NMDA receptor

Since glycine was first shown to potentiate glutamate responses at the NMDA receptor (Johnson and Asher, 1987) there has been an increasing amount of evidence to suggest that allosteric interactions occur between the glutamate and glycine recognition sites. Radioligand binding studies have shown that glycine can increase \[^{3}H\] glutamate binding and inhibit the binding of the glutamate antagonist, \[^{3}H\] AP5, to the NMDA receptor (Monaghan et al., 1988). This allosteric action of glycine on the binding of both \[^{3}H\] glutamate and \[^{3}H\] AP5 was reconciled as a change in the affinity of these ligands for the glutamate binding site on the NMDA receptor by glycine (Monaghan et al., 1988). L-Glutamate is also reported to enhance the binding of glycine to the co-agonist glycine binding site (Monaghan et al., 1988). However, many reports claim that NMDA receptor agonists and antagonists can either enhance or inhibit the radioligand binding of both \[^{3}H\] glycine and the glycine site antagonist, \[^{3}H\] 5,7 DCKA (Kessler et al., 1989; Baron et al., 1991). These findings are complicated further as different glutamate antagonists have differential effects on \[^{3}H\] glycine radioligand binding. Those glutamate antagonists with five carbon atoms linking the carboxyl and phosphonate moieties i.e., AP5 and CGS 19755, partially inhibit \[^{3}H\] glycine binding whereas antagonists with seven carbon atoms linking the carboxyl and phosphonate moieties
INTRODUCTION

(CPP) have little effect on [3H] glycine binding per se (Grimwood et al., 1993). The significance of these complex interactions between the two amino acid recognition sites on the NMDA receptor remains to be elucidated.

1.5 PHARMACOLOGICAL EVIDENCE FOR REGIONAL HETEROGENEITY OF NMDA RECEPTORS

Prior to the advent of molecular biology, many independent studies pointed towards the existence of distinct NMDA receptor subtypes in the CNS. For example, autoradiographic studies showed that [3H] glutamate labelled preferentially the septum and striatum whereas the NMDA antagonist, [3H] CPP, labelling was mostly detected in the thalamus and the cerebral cortex (Monaghan et al., 1988). These two populations were termed agonist- and antagonist-preferring. Radioligand binding studies confirmed these findings as the agonist-preferring population of receptors displayed higher affinities for NMDA agonists and the converse was true for antagonist-preferring receptors (Monaghan et al., 1988). A third subclass of NMDA receptor was also identified which exhibited distinctive pharmacological characteristics compared to forebrain NMDA receptors including a low affinity for glutamate site ligands, polyamines and channel blockers (Beaton et al., 1992). This group comprised cerebellar NMDA receptors. Finally, midline thalamic receptors were characterised as the fourth subclass of NMDA receptors which displayed a distinct difference in their affinity for both the NMDA site antagonist; 6-tetrazole-decahydroisoquinoline-3-carboxylic acid.
(LY233536) and MK801 compared with cerebellar and forebrain NMDA receptors (Beaton et al., 1992).

1.6 MOLECULAR BIOLOGY OF NMDA RECEPTORS

Expression cloning in Xenopus oocytes led to the isolation of the first member of the NMDA receptor family, the clone, λN60, from a rat forebrain complementary deoxyribonucleic acid (cDNA) library encoding the NR1 subunit (Moriyoshi et al., 1991). In the following year after the cloning of the rat NR1, the mouse counterpart was cloned by Yamakaki et al., (1992b) and was termed, ζ1. Four additional genes encoding the NR2A, NR2B, NR2C and NR2D (mouse NR2 subunits are termed ε1-ε4 respectively) NMDA receptor subunits were also identified by low stringency screening and polymerase chain reaction (PCR) amplification of rat and mouse forebrain cDNA libraries (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Ishii et al., 1993; Monyer et al., 1992). Recently, both Ciaberra et al., (1995) and Sucher et al., (1995) independently isolated the clone, NMDAR-L (χ1) which was originally described as an NMDA receptor-like subunit but has been confirmed as an NMDA receptor subunit; NR3A (Das et al., 1998).

The NR1 subunit was predicted to have 938 amino acids with a calculated molecular mass (Mr) of 105 kilodaltons (kDa). The proposed mature NR2A-NR2D subunits were composed of 1445, 1456, 1220 and 1296 amino acids with calculated Mrs of 163, 162, 133 and 141 kDa respectively. The NR3A polypeptide was predicted to comprise of
1115 amino acids with a calculated Mr of 125 kDa. Figure 1.4 shows a schematic representation of the adult rat NMDA receptor subunits with their number of amino acids residues.

The NR2A-2D subunits share ~50-70% amino acid sequence identity with each other but only ~15% amino acid sequence identity with the NR1 subunit. The primary structure of the NR3A subunit revealed a family relation of ~27% amino acid sequence identity to the previously characterised NMDA receptors subunits. Predicted amino acid sequences for the mature polypeptides of the rat NR1 subunits have ~99.8% amino acid sequence identity with their mouse counterparts whereas the rat and mouse NR2 protein sequences are more diverse sharing 88% amino acid sequence identity with their mouse counterparts. Both NR1 and NR2 subunits display conserved amino acid sequence identity with the AMPA and kainate receptors (11-16%). The NR3A subunit shows ~24% amino acid sequence identity with non-NMDA ionotropic receptor subunits (Sucher et al., 1995).

The topological organisation of the ionotropic receptor subunits is discussed in section 1.7. Briefly, the NMDA receptor subunits are believed to follow a three transmembrane model with both the amino-terminus and the domain between M3 and M4 located on the extracellular side of the cell membrane with an intracellular carboxyl-terminus. In contrast, the M2 domain forms a monotopic hinge region which is thought to line the lumen of the ion channel (Burnashev et al., 1992a).

The deduced amino acid sequence of the NR1 subunit showed several interesting features including an asparagine (N) residue in the putative channel forming region, M2.
Homologous to the NR1 subunit, NR2 subunits contain an N residue in the M2 channel forming domain. However, when the M2 region of the NR3A subunit was aligned with the other NMDA receptor subunits where N residues are located a glycine residue was found in the homologous position (Sucher et al., 1995). In contrast to the NR1 and NR2 receptor subunits, ionotropic non-NMDA receptor subunits contain a glutamine (Q) or arginine (R) residues in the homologous position as discussed in section 1.13. Furthermore, the amino terminus of the NR1 subunit was surprisingly large comprising of half of the mass of the polypeptide. In contrast to the NR1 subunit and other subunits of the ionotropic glutamate receptor family, both the NR2A and NR2B subunits have long carboxy terminal extensions of greater than 600 amino acids (Monyer et al., 1992). The cloning of NR1 receptor subunit splice variants was reported by Anatharam et al., (1992), Durand et al., (1992), Nakanishi et al., (1992), Sugihara et al., (1992), Durand et al., (1993) and Hollmann et al., (1993). It is now known that the NR1 subunit gene has a total of 22 exons, three (exons 5, 21 and 22) of which undergo differential alternative splicing to yield eight isoforms (Hollmann et al., 1993). A diagrammatic representation of the NR1 splice variants are shown in Figure 1.5. Exon 5, termed N1, encodes a splice cassette of 21 amino acids inserted in the predicted amino-terminus domain of the NR1 protein and is found in the splice forms NR1-1b, NR1-2b, NR1-3b and NR1-4b. Exons 21 and 22 encode two independent consecutive splice variants of 37 (C1) and 38 (C2) amino acids in the last stretch of the carboxy-terminus domain of the NR1 subunit. C1-containing splice forms are present in NR1-1a, NR1-1b, NR1-3a and NR1-3b whereas the C2-containing isoforms are found in NR1-1a, NR1-1b, NR1-2a
and NR1-2b. Splicing out the exon segment that encodes the C2 insert removes the first stop codon resulting in a new open reading frame that encodes an unrelated sequence of 22 amino acids (C2'), present in NR1-3a, NR1-3b, NR1-4a and NR1-4b (reviewed in Zukin and Bennett, 1995). The nomenclature used for the eight splice variants, i.e., NR1-1a-NR1-4b is after Nakanishi et al., (1992). Consequently, the first rat NR1 subunit to be cloned which lacked the N1 exon but contained both C1 and C2 exons is denoted NR1-1a and the first ζ1 subunit cloned again lacked the N1 exon but contained the C2 exon is termed NR1-2a.

Multiple splice forms of the rodent, human NR2C subunits and the rat NR2D have also been described (Suchanek et al., 1995; Daggett et al., 1998).

1.7 DECIPHERING THE TRANSMEMBRANE TOPOLOGY OF NMDA RECEPTORS

By way of an example, the proposed transmembrane topology of the NMDA receptor subunits is discussed below but it is also analogous to the AMPA and kainate receptor subunits (Hollmann et al., 1994). Early models based on hydrophobicity profiles of NMDA receptors subunits predicted four transmembrane segments, M1-M4, thus adopting the transmembrane topology typical of nicotinic acetylcholine, glycine and γ-aminobutyric acid (GABA)-gated channels (Hollmann et al., 1989). This topological organisation placed the amino-terminus domain and the carboxyl-terminus region of the NMDA receptors subunits on the extracellular receptor surface. However, experimental
results from a variety of studies soon challenged such a model. Firstly, site-directed mutagenesis experiments identified several amino acids in the NR1 subunit involved in the putative pharmacophore of glycine, these regions are two discontinuous segments of approximately 150 amino acids termed S1 and S2 (Kuryatov et al., 1994; Hirai et al., 1996; Uchino et al., 1997; Wood et al., 1997). S1 is located on the extracellular region preceding the first transmembrane segment M1 and S2 is found between the putative M3 and M4 regions. As discussed in section 1.8, glycine binding to the NMDA receptor requires the presence of both S1 and S2, these domains must be therefore located extracellular which contradicts the earlier model which would have placed S2 intracellular to the cell surface. A second study arguing against the four transmembrane model was the finding that putative phosphorylation sites located on the carboxyl-terminus of the NR1 subunit are phosphorylated in both cortical and transiently transfected cells (Tingley et al., 1993). This report proposed that the carboxyl-terminus of the receptor must be located intracellularly. However, the possibility of extracellular phosphorylation due to protein kinases located outside the cell surface cannot be ignored (Erlick, 1996). The intracellular location of the carboxyl-terminal of the NMDA receptor was unequivocally demonstrated when this region was found to interact with cytosolic post-synaptic density (PSD)-associated proteins containing PSD 95/ discs large/ zonula occuludentes (PDZ) motifs (Kornau et al., 1995). This clearly suggests that the carboxyl terminal is located intracellularly and not extracellularly. The strongest evidence disputing the four transmembrane topology model arises from studies by Hirai et al., (1996). This group used epitope tagging to demonstrate that the amino-terminal
domain and the domain between M3 and M4 regions are indeed extracellular whereas the carboxyl-terminal is intracellular. The results from these studies support a three transmembrane structure, where the M2 hydrophobic region does not transverse the plasma membrane as suggested by the earlier model but forms a re-entrant loop within the membrane (Hirai et al., 1996). The M2 region of NMDA receptor subunits has been proposed to be analogous to that of the pore-forming segment (P segment) of K⁺-gated channels (Wo and Oswald, 1995). Both the original and current model of the topology of ionotropic glutamate receptor subunits is shown in Figure 1.6.

1.8 LOCALISATION OF GLUTAMATE AND GLYCINE BINDING SITES ON CLONED NMDA RECEPTORS

As stated above, the NR1 subunit appears to contain many determinants that constitute the glycine binding site of NMDA receptors. This is suggested by radioligand binding studies that have demonstrated that [³H] glycine and various radiolabelled glycine site antagonists bind to NR1 subunits expressed alone in mammalian cells (Grimwood et al., 1995; Siegel et al., 1996; Chazot et al., 1998). The pharmacological profiles of these glycine site antagonist binding to recombinant NR1 subunits were indistinguishable to antagonist binding to native receptors. In agreement with radioligand binding studies, mutational analysis also suggested that the major molecular determinants for glycine binding reside on the NR1 subunit (Kuryatov et al., 1994). For example, site-directed mutagenesis studies have shown that the glycine binding domain is located on the S1
and S2 segments of the NR1 subunit (Hirai et al., 1996) (section 1.7). The findings of these studies were further reinforced by Ivanovic et al., (1998). This latter group created a soluble protein by directly connecting two DNA stretches that encode S1 and S2 segments of the NR1 subunit upon expression using a baculovirus system. The pharmacological profile of the S1-S2 protein was similar to wild-type NR1 subunits expressed in mammalian cells. More recently, the photoaffinity labelled glycine site antagonist, [^3]H]CGP 61594, labelled predominantly the NR1 subunit (Honer et al., 1998).

The glutamate binding site is proposed to be located on the NR2 subunit. This is supported by radioligand binding studies that have shown that [^3]H] glutamate binds to the NR2A subunit expressed in HEK 293 cells (Kendrick et al., 1996). In addition, in vitro mutagenesis studies also identified amino acid residues within NR2 subunits that are important in the binding site of glutamate (Laube et al., 1997).

1.9 DISTRIBUTION OF NMDA RECEPTOR SUBUNITS IN THE CNS

Studies employing in situ hybridisation or immunochemical approaches have demonstrated that the respective NMDA receptor subunits in the developing and adult brain are differentially expressed in specific regions in the CNS.
1.9.1 Distribution of the NMDA receptor subunits mRNAs

The mRNA encoding the NR1 subunit is distributed ubiquitously in the immature and adult brain. However, each of the NR1 splice variants displayed regional variations in both the developing and adult CNS. For example, expression of the N1-containing splice forms were found predominantly in the hippocampal pyramidal CA3 cells, the thalamic region and in the granule layers of the cerebellum (Standaert et al., 1994). In contrast, oligonucleotide probes directed towards C1- and C2-containing variants showed prominent labelling throughout the hippocampus, striatum and the granule layer of the cerebellum (Laurie and Seeburg, 1994). However, the expression of C2'-containing isoforms was very sparse and was only found in the hippocampus and the cortex (Laurie and Seeburg, 1994). In general, there is considerable overlap in the spatial distribution of the different NR1 splice variants studied.

The expression of the respective NR2 and NR3A subunit mRNAs are differentially regulated during development of the rodent brain. In contrast to the wide distribution of the NR2B subunit mRNA in the embryonic brain, the NR2D subunit mRNA is found exclusively in the brainstem and diencephalon whereas the mRNA encoding the NR3A subunit is pronounced in the cortex, thalamus, brainstem, cerebellum and the spinal cord. By postnatal day 14, the expression of the NMDA receptor subunit mRNAs changes drastically. For example, the mRNA expression of both NR2D and NR3A declines. The mRNA for the NR2D subunit remains only in the thalamus while the NR3A mRNA transcripts are predominantly detected in the nucleus of the olfactory tract.
(Sucher et al., 1995). The mRNA encoding the NR2B subunit becomes restricted to the forebrain (Monyer et al., 1994). In contrast, the NR2A mRNA is found distributed throughout the entire brain whereas mRNA encoding the NR2C subunit is expressed and found principally in the cerebellum (Watanabe et al., 1992, 1993).

1.9.2 Distribution of the NMDA receptor subunits

Distribution of levels of NMDA receptor subunit mRNA accrued from in situ distribution studies does not clearly define expression of the resultant protein. Immunochemical approaches have been used to study the expressional profiles of the NMDA receptor subunits at the protein level. Immunocytochemical localisation of the NR1 subunit demonstrated it to be prominent in all brain regions in agreement with the results from in situ hybridisation studies (Brose et al., 1993). However, different splice variants of the NR1 subunit have shown distinct expression patterns as demonstrated by immunocytochemistry using the respective subunit-specific antibody. For example, C1-containing forms were found in the hippocampal region whereas N1-containing variants were expressed in the subthalamic nucleus of the basal ganglia (Standaert et al., 1994).

In close agreement with the expression profiles determined by in situ hybridisation, the NR2A subunit protein was found abundantly distributed throughout the adult brain with the highest expression levels in the hippocampus, cerebral cortex, thalamus, cerebellum and striatum (Wenzel et al., 1995; Luo et al., 1997). The NR2B subunit protein was
predominantly expressed in the hippocampus, cerebral cortex, striatum and olfactory bulb (Luo et al., 1997; Charton et al., 1999). In contrast, the NR2C subunit protein was almost entirely expressed in the cerebellum (Wenzel et al., 1995) while the NR2D subunit protein was restricted to the spinal cord, brain stem regions, striatum, thalamic regions and the olfactory bulb (Dunah et al., 1998).

Thus these studies using either in situ hybridisation or immunochemical approaches have shown that native NMDA receptor subunits have overlapping temporal and spatial distributions within the CNS, suggesting that the native receptor comprises of co-associating receptor subunits.

1.10 STOICHIOMETRY OF THE NMDA RECEPTOR COMPLEX

As stated above, studies employing either in situ hybridisation or immunochemical approaches suggest that native NMDA receptors are hetero-oligomeric complexes. However, the elucidation of the receptor's structure is hampered due to its resistance to detergent extraction from the membrane in the native form. Nonetheless, biochemical studies have implied that in the rat brain and transfected cell lines that functional NMDA receptors are formed from oligomeric complexes of NR1 and NR2 subunits although the precise stoichiometry of these complexes remains ambiguous (Chazot et al., 1994; Sheng et al., 1994; Blahos and Wenghold, 1996). In support of the biochemical studies, functional investigations into the stoichiometry of the NMDA receptor have indicated that the binding of at least two molecules of both glutamate and
glycine are required for receptor activation (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). The glutamate and glycine binding sites have been localised to the NR2 and NR1 subunits respectively by mutagenesis experiments as discussed in section 1.8 (Kuryatov et al., 1994; Hirai et al., 1996; Laube et al., 1997), thus suggesting that the receptor complex must be at least a tetramer. In favour of a tetrameric NMDA receptor structure as discussed above, voltage-gated K⁺ channels are analogous to NMDA receptors in having re-entrant loop domains, i.e. P-domain, and are known to be tetramers (MacKinnon, 1995).

However, studies using chemical cross-linking and size exclusion chromatography have estimated the size of the native NMDA receptors to be ~730 kDa (Brose et al., 1993). Given the size of the NR1 (115 kDa) and NR2 (~160-180 kDa) these results are consistent with a pentameric stoichiometry in analogy with the nicotinic acetylcholine receptor. Furthermore, the complexity of the NMDA receptor subunit arrangement begins to unfold when the results from immunopurification studies of native NMDA receptors are considered. These respective immunopurification studies using native NMDA receptors have identified more than one NR1 splice variant within the same receptor complex. In addition to heterogeneity resulting from more than one NR1 splice form comprising a receptor oligomer, at least two different NR2 subunits co-associated with an NR1 subunit has also been reported (Sheng et al., 1994; Chazot and Stephenson, 1997a). However, these receptors represent a minor population (Chazot and Stephenson, 1997a).

Comparative studies of recombinant and native NMDA receptors can provide further
insight to information regarding the stoichiometry of the NMDA receptor. Co-expression of NR1-1a with NR2A and NR2C subunits in *Xenopus* oocytes was the first indication that the NR1-1a could assembly with more than one type of NR2 complex (Wafford *et al.*, 1993). This is because the assembled receptor had an affinity for glycine that was distinct from that of NR1-1a/NR2A and NR1-1a/NR2C combinations, suggesting the formation of the NR1-1a/NR2A/NR2C complex. Subsequent biochemical experiments using HEK 293 cells transfected with NR1-1a, NR2A and NR2C clones demonstrated that the NR1-1a/NR2A/NR2C receptor had pharmacological properties in line with some native cerebellar NMDA receptors (Chazot *et al.*, 1994). However, no direct conclusion of the subunit arrangement of NMDA receptors can be ascertained from the above reports.

Recently, the exact copy number of NR1 and NR2 subunits per oligomer was investigated by the co-expression of wild-type and mutant forms of either the NR1 or NR2 subunits (Béhé *et al.*, 1995; Premkumar and Auerbach, 1997; Laube *et al.*, 1998). The resultant channels displayed either wild-type, mutant or hybrid wild-type/mutant properties. The relative proportions of the three different receptor populations changed with variations to the ratio of wild-type to mutant subunits. Analysis of resulting dose-response curves of the three different receptor populations predicted a tetrameric structure for the NMDA receptors (Laube *et al.*, 1998). In contrast, Premkumar and Auerbach, (1997), using a similar strategy to Laube *et al.*, (1998) postulated a pentameric structure of the NMDA receptor composed of three NR1 and two NR2 subunits. Reinforcing the argument by Premkumar and Auerbach, (1997), a recent study
employing an immunobiochemical approach suggested that the NMDA receptor complex was at least a pentameric structure (Hawkins et al., 1999). However, in contrast to Premkumar and Auerbach, two NR1 and three NR2 subunits per oligomer were proposed.

In summary, all the combined results reported from different groups are at variance with each other and are far from conclusive in regard to the stoichiometry of the NMDA receptor.

1.11 MOLECULAR ARCHITECTURE OF THE GLUTAMATE AND GLYCINE BINDING SITES

Detailed amino acid sequence of the ionotropic glutamate receptor subunit families with various bacterial periplasmic binding proteins (PBPs) led Nakanishi et al., (1990) and O'Hara et al., (1993) to recognise that several regions of amino acid sequence similarities exist between these protein families. PBPs are monomeric soluble proteins that are found in the periplasmic space of gram-negative bacteria and include leucine/isoleucine/valine-binding protein (LIVBP), glutamate binding protein (QBP) and lysine/arginine/ornithine-binding protein (LOABP). It was proposed that the first 400 amino acids of ionotropic glutamate receptors share a common folding motif with LIVBP (reviewed in Pass, 1998). In addition, the 150 amino acids of both S1 and S2 domains of ionotropic glutamate receptors (section 1.8) were structurally related to the QBP and LOABP (reviewed in Pass, 1998). Domain analogy between bacterial PBPs
and ionotropic glutamate receptors is shown in Figure 1.7.

X-ray crystallography studies have shown that different PBPs fold to a similar three-dimensional (3D) structure which consists of two distinct globular domains that are interconnected by a hinge region. The two lobes are separated by a deep cleft in which the ligand is accommodated (Quiocho and Ledvina, 1996). Attracted by the idea that S1 and S2 domains might share a similar crystalline structure with PBPs several groups have undertaken to identify residues involved in the ligand binding to NMDA receptors subunits (Kuryatov et al., 1994; Wafford et al., 1995; Hirai et al., 1996). For example, Hirai et al. (1996) identified phenylalanine F309, F392, F735 and F736 as key residues involved in glycine binding to the NR1 subunit by in vitro mutagenesis. Residues F735 and F736 located within the S2 domain of the NR1 subunit corresponded to Y190 and F191 respectively in the second connecting strand of LOABP. These residues are considered pivotal for the opening and closing of the ligand binding pockets of LOABP whereas aromatic residues in the NR1 protein, F390 and Y392, located within the S1 domain corresponds to residues in lobe 1 of LOABP. Based on this model, it is proposed that the binding of glycine causes the two lobes containing the S1 and S2 domains to come in contact with each other that would induce a closed conformation of the ligand binding pocket (Figure 1.8).

Mutations of aromatic residues in the NR1 subunit, F390 and Y392, drastically decreased the affinity of a glycine site antagonist, 7-CLKA. In contrast, the mutants NR1F735A and NR1F736A did not effect the affinity of 7-CLKA for the NR1 subunit (Hirai et al., 1996). This suggested that the glycine antagonist binding domain could be
exclusively located on the S1 domain of the NR1 subunit and that antagonist binding to this region may prevent the closing movement of the ligand binding pocket by steric hindrance.

The proposed model of glycine binding site based on LOABP also extends to the glutamate binding site on the NR2 subunit as residues identified to be crucial for glutamate binding on the NR2B subunit were at analogous positions to various substrate binding residues of LOABP (Laube et al., 1997). In addition, the crystalline structure of the rat GluR2 S1 S2 'flop' isoform bound to the non-desensitising agonist, kainate was recently solved by multiwavelength anomalous diffraction (Armstrong et al., 1998). The resolved structure of the AMPA receptor subunit, GluR2 subunit (section 1.13.1) was similar to that of the QBP.

... 'our complicated experiments have no longer anything to do with nature in her own right, but with nature changed and transformed by our own cognitive activity' - Werner Hesienberg (1901-1976)

1.12 PHARMACOLOGICAL AND FUNCTIONAL PROPERTIES OF RECOMBINANT NMDA RECEPTORS

Investigation of the pharmacological and biophysical properties of recombinant NMDA receptors has aided in the dissection of the subunit complements of the native receptor.
1.12.1 Pharmacological properties of NR1 subunits

Expression of the rat NR1-la subunit in *Xenopus* oocytes was shown to form functional homomeric channels with characteristic properties resembling native NMDA receptors (Moriyoshi *et al.*, 1991). These properties include activation by glutamate and NMDA in the presence of glycine, permeability of Ca\(^{2+}\) ions, voltage-dependent Mg\(^{2+}\) blockade and block by the open channel blocker, MK801. However, studies characterising the pharmacology of NR1-la receptors have concluded that this subunit expressed alone in mammalian expression systems does not assemble into functional homomeric complexes (Ishmael *et al.*, 1996). This was demonstrated by the lack of specific radioligand binding of \(^{3}H\) MK801 and no detectable Ca\(^{2+}\) ion flux in HEK 293 cells expressing only NR1-la subunits. These findings agreed with electrophysiological experiments that find no detectable currents in mammalian cells transiently or stably expressing the NR1-la subunit alone (Monyer *et al.*, 1992; Lynch *et al.*, 1993). The reason for the discrepancy between the two expression systems may be due to oocytes endogenously expressing a glutamate receptor subunit, XenU1 which could assemble with the NR1-la subunit to give functional homomeric receptors (Soloviev and Barnard, 1997).

However, there are conflicting reports to whether open channel blockers bind to the NR1-la subunits expressed in mammalian cells (Chazot *et al.*, 1992; Laurie and Seeburg, 1994; Grimwood *et al.*, 1995; Ishmael *et al.*, 1996). For example, both Chazot *et al.*, (1992) and Laurie and Seeburg, (1994) detected high affinity \(^{3}H\) MK801
radioligand binding to NR1-1a subunits in membranes derived from HEK 293 cells transiently transfected with the NR1-1a clone. Differences between these reports and others may be explained by variations in the radioligand binding procedure as discussed in section 4.2.

Analysis of the properties of NR1 subunit splice variants expressed in *Xenopus* oocytes and mammalian cells revealed that the splice forms exhibit differing pharmacological characteristics (Durand *et al.*, 1993; Hollmann *et al.*, 1993; Zhang *et al.*, 1994). For example, NMDA receptors assembled from NR1 splice forms lacking the N1 exon exhibited a five-fold higher affinity for glutamate and showed greater potentiation by spermine at high glycine concentrations compared with N1-containing splice forms (Durand *et al.*, 1993; Hollmann *et al.*, 1993). Site-directed mutagenesis experiments have demonstrated that there is a net increase in positive charge conferred by the insertion of the N1 exon in the NR1 subunit which may be responsible for the polyamine insensitivity of N1-containing splice forms (Zheng *et al.*, 1994). However, at low glycine concentrations all NR1 subunit isoforms could be potentiated by spermine. This form of potentiation occurs by an increase in the receptor affinity for glycine (Zhang *et al.*, 1994). These differential effects of spermine potentiation exhibited at low but not at high concentrations of glycine suggests that the two actions of spermine are independent and might be mediated at separate sites on the NR1 subunit.

The isoforms also differed in the extent to which they could be potentiated by protein kinase C (PKC) and Zn$^{2+}$. For example, N1-containing splice forms showed reduced
potentiation by Zn\(^{2+}\) and an ~seven-fold greater potentiation by PKC compared with Cl-containing splice variants (Nakanishi \textit{et al.}, 1992; Durand \textit{et al.}, 1993; Zheng \textit{et al.}, 1994).

Furthermore, studies have shown that the NMDA receptor can be inhibited by protons (Traynelis and Cull-Candy, 1990). The pH sensitivity of the receptor is directly influenced by the type of NR1 splice form present in the receptor complex such that variants that contain the N1 exon are less sensitive to proton inhibition than are splice forms that lack this insert (Traynelis \textit{et al.}, 1995).

Previously, it has been shown that the glycine binding site has been localised to the NR1 subunit (section 1.8). Radioligand binding studies have shown that all eight different NR1 splice forms have similar affinities for \(^{3}\text{H}\) glycine (Durand \textit{et al.}, 1992). In regard to glycine site antagonists, \(^{3}\text{H}\) MDL105,519 exhibited similar affinities for NR1-1a and NR1-2a subunits expressed in HEK 293 cells (Chazot \textit{et al.}, 1998) whereas another glycine site antagonist, \(^{3}\text{H}\) L689,560, displayed a two-fold lower affinity for NR1-1a compared with NR1-4a subunits expressed in HEK 293 cells (Grimwood \textit{et al.}, 1995).

1.12.2 Pharmacological properties of heteromeric NMDA receptors

Members of the NR2 subunit family do not form functional ion channels when expressed alone in \textit{Xenopus} oocytes or mammalian cell systems (Monyer \textit{et al.}, 1992). Co-expression of the NR1-1a subunit with an NR2 subunit elicited large responses in oocytes to glutamate or NMDA compared with the responses detected by the NR1-1a
subunit alone. Similar to the NR2A subunit, injection of NR3A cRNA into *Xenopus* oocytes did not lead to the expression of homomeric glutamate-activated channels. Although co-injection of the triple combination of NR3A with NR1 and NR2B cRNAs led to a decrease in the current magnitude compared with currents obtained after the co-expression of NR1/NR2B heteromeric receptors (Sucher *et al.*, 1995). As stated above, these findings taken together suggest that highly active NMDA receptor channels in both oocytes and in mammalian cells are formed from the co-assembly of an NR1 and NR2 receptor subunits (Meguro *et al.*, 1992; Monyer *et al.*, 1992; Ishii *et al.*, 1993).

As with NR1 subunit splice forms, it has become clear from expression studies that the pharmacological properties of NMDA receptors are influenced by the type of NR2 subunit present in the heteromeric receptor complex.

### 1.12.2.1 Glutamate and glycine affinities of heteromeric NMDA receptors

Heteromeric recombinant NMDA receptors exhibit differences in their affinity for both glutamate and glycine. For example, radioligand binding studies demonstrated that combinations comprising of NR1-1a/NR2B receptors expressed in HEK 293 cells have a modest ~two-fold higher affinity for \[^{3}H\] glutamate compared with NR1-1a/NR2A complexes (Laurie and Seeburg, 1994). In accordance with these results, the affinity for glutamate and NMDA in receptor combinations expressed in oocytes was found to be higher for NR1-1a/NR2B and NR1-1a/NR2C compared to NR1-1a/NR2A subunit combinations (Monyer *et al.*, 1992). In contrast to the \[^{3}H\] glutamate selectivity of
heteromeric NMDA receptors, glutamate antagonists exhibited the reverse profile by having higher affinity for NR2A-containing receptors. This was shown by competition experiments between $[^3]H$ glutamate radioligand binding to heteromeric NR1-1a/NR2 combinations expressed in HEK 293 cells by various NMDA site competitive antagonists including APV, D-CPP-ene and CGS19755 (Laurie and Seeburg, 1994). For each of these NMDA receptor antagonists, NR1-1a/NR2A receptors exhibited the highest affinity in comparison to NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D complexes (Laurie and Seeburg, 1994). Analogously, the displacement of $[^3]H$ glutamate radioligand binding by another competitive NMDA receptor site antagonist, D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid (CGP39653) could only be detected in NR1-1a/NR2A subunit combinations (Laurie and Seeburg, 1994; Lynch et al., 1993).

The absolute affinity of glycine for NMDA receptors is relatively difficult to determine from both electrophysiological and radioligand binding assays due to the variable levels of endogenous glycine contamination (Berger et al., 1995). However, most electrophysiological studies report that the rank order of increasing affinity for glycine in receptor combinations expressed in oocytes is NR1-1a/NR2D > NR1-1a/NR2C > NR1-1a/NR2B > NR1-1a/NR2A (Kutsuwada et al., 1992; Wafford et al., 1993; Priestley et al., 1995; Buller and Monaghan, 1997). One notable exception using radioligand binding assays showed that the rank order of glycine affinity determined by the inhibition of $[^3]H$ 5,7 DCKA radioligand binding for receptor combinations expressed in HEK 293 cells was NR1-1a/NR2C > NR1-1a/NR2D = NR1-1a/NR2B >
NR1-1a/NR2A (Laurie and Seeburg, 1994). The molecular basis for this selectivity has not been fully elucidated but is probably related to allosteric interactions between the NR2 subunits and the glycine recognition site on the NR1 subunit.

In contrast to the subtype-selectivity reported for glycine, most glycine site antagonists exhibit no or moderate differentiation between heteromeric NMDA receptors, i.e. NR1/NR2s. For example, electrophysiological studies showed that the glycine site antagonist, ACEA 1011 displayed similar affinities for heteromeric NMDA receptors, i.e., NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D expressed in oocytes (Woodward et al., 1995). However, electrophysiological studies demonstrated that L689,560 has a five-fold selectivity for human NR1-1a/NR2A compared with NR1-1a/NR2B receptors (Hess et al., 1996). More recently, it was shown that the photoaffinity labelled glycine site antagonist, [3H] CGP61594 expressed a ten-fold higher affinity for NR1-1a/NR2B-containing receptors compared with NR1-1a/NR2A, NR1-1a/NR2C and NR1-1a/NR2D complexes expressed in HEK 293 cells (Honer et al., 1998).

1.12.2.2 Channel properties of heteromeric NMDA receptors

The expression of recombinant receptors in mammalian and *Xenopus* oocytes along with site-directed mutagenesis experiments has provided an insight to the molecular basis for the Mg$^{2+}$ voltage-dependent block of NMDA receptors. For example, recombinant NR1-1a/NR2A and NR1-1a/NR2B receptors were shown to exhibit a more prominent Mg$^{2+}$
block compared with NR1-1a/NR2C and NR1-1a/NR2D channels (Monyer et al., 1994). The cationic selectivity of these channels is due in part to the presence of an N residue located within the M2 segment that constitutes the ion channel pore of NMDA receptors (section 1.6) (Burnashev et al., 1992a). This was demonstrated by site-directed mutagenesis experiments that showed that both \(NR1^{NS98Q}/NR2A\) and \(NR1/NR2A^{NS95Q}\) mutant receptors exhibited reduced sensitivity to \(Mg^{2+}\) block in comparison to the wild-type receptor channel. However, studies have suggested that the N residues of NR1 and NR2A subunits are located at non-homologous positions in the NMDA receptor channel pore. Consequently, further site-directed mutagenesis studies were performed to identify the structural determinants that define the selectivity of the ion channel pore (Wollmuth et al., 1996). Findings from these experiments identified residues on both NR1 and NR2A subunits that confer ion channel selectivity, but these residues were not at equivalent depths within the channel. For example, Williams and co-workers showed that mutating tryptophan residues in the M2 region of \(NR2B\) (W607) and \(NR2A\) (W606) for non-aromatic amino acids reduced the \(Mg^{2+}\) blockade whereas the homologous mutations in the NR1 subunit had no effect the on \(Mg^{2+}\) blockade (Williams et al., 1998).

In contrast to the extracellular \(Mg^{2+}\) block discussed above and earlier (section 1.44), Johnson and Ascher in 1990, proposed the existence of a second \(Mg^{2+}\) ion binding site located at an intracellular site within NMDA receptor channel pore. \textit{In vitro} mutagenesis experiments confirm that both intracellular and extracellular \(Mg^{2+}\) block occurs at distinct binding sites. This was demonstrated by mutating the glycine G618 residue of
The NR1 subunit which reduced the external Mg\(^{2+}\) block of heteromeric NMDA receptors but had no effect on the block by internal Mg\(^{2+}\). Conversely, mutations at Q621 of the NR1 subunit appear to influence internal Mg\(^{2+}\) block of heteromeric receptor complexes without effecting the external Mg\(^{2+}\) block (Kupper et al., 1996; Wollmuth et al., 1998).

Different heteromeric NR1-1a/NR2 receptors have exhibited distinct calcium ion permeabilities. For example, Grant et al., (1997) demonstrated that agonist application to NR1-1a/NR2A or NR1-1a/NR2B transfected HEK 293 cells elicited robust rises in intracellular levels of Ca\(^{2+}\) ions using the calcium sensitive bioluminescent protein, aequorin, followed by single cell imaging with the fluorescent calcium indicator, fluo-3. In contrast, no detectable intracellular Ca\(^{2+}\) responses were observed in cells expressing the NR1-1a/NR2C receptor (Grant et al., 1997). Furthermore, expression of NR1-1a/NR2A and NR1-1a/NR2B receptors in HEK 293 cells results in cell cytotoxicity, which is not apparent in cells expressing heteromeric NR1-1a/NR2C channels (Cik et al., 1993; Chazot et al., 1994). These results may be explained by a low Ca\(^{2+}\) ion permeability of NR1-1a/NR2C channels compared with NR1-1a/NR2A and NR1-1a/NR2B receptors since Ca\(^{2+}\) ion overload has been implicated in cytotoxicity (section 1.15.4) (Grant et al., 1997). However, Ca\(^{2+}\) permeability estimated by measuring reversal potentials were comparable among all four NR1-1a/NR2 heteromeric channels (Monyer et al., 1994). As yet, the significance of the distinct calcium ion permeabilities exhibited by the different NMDA heteromeric NR1/NR2 receptors has not been fully elucidated.
INTRODUCTION

In many cases the functional and pharmacological properties of recombinant NMDA receptors have correlated with native NMDA receptors which has proved useful in the dissection of the subunit composition of native receptors. For example, NR1-1a/NR2A and NR1-1a/NR2B heteromeric receptors expressed in Xenopus oocytes exhibited principle conductances of 40 pS and 50 pS similar to hippocampal CA1 pyramidal cells (Stern et al., 1992). The single channel characteristics of the NR1-1a/NR2C channels with conductances of 19 pS and 36 pS were markedly similar to the native channels of the large cerebellar granule cells whereas NR1-1a/NR2D receptor's single channel properties were reciprocated by neonatal Purkinje cells which include low-conductances, extended opening times, low sensitivity to Mg\(^{2+}\) and offset decay time constants of ~5000 ms (Cull-Candy et al., 1998).

1.12.2.3 Pharmacological differences in heteromeric NMDA receptors for channel blockers

Previously it was discussed that the NR1 subunit alone is not sufficient for reconstitution of the \(^{[3]H}\) MK801 binding site (1.12.1). However, site-directed mutagenesis experiments have identified amino acids on the NR1-1a subunit including N598 and N619 that are essential for MK801 to block conductances in NR1-1a/NR2 heteromeric receptors (Burnashev et al., 1992a; Sakurada et al., 1993). In support of this, Sonders et al., (1990) have shown that an azido derivative of MK801 specifically photolabels the NR1 subunit in rat membranes. Therefore, it appears that this subunit
is still likely to make an essential contribution to non-competitive antagonist binding domains within the NMDA receptor channel.

Furthermore, studies have shown that MK801 exhibits marked subunit-selectivity between channels comprising of NR1-1a/NR2 receptors. For example, in radioligand binding studies, \(^{3}H\) MK801 displayed ~25-fold higher affinity for NR1-1a/NR2A and NR1-1a/NR2B compared with NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells (Laurie and Seeburg, 1994). These radioligand binding results correlated with findings from electrophysiological experiments where MK801 inhibited glutamate-evoked currents of NMDA heteromeric receptors expressed in oocytes with a higher affinity for NR1-1a/NR2A, NR1-1a/NR2B compared to NR1-1a/NR2C and NR1-1a/NR2D receptors (Yamakura et al., 1993). However, both ketamine and PCP did not differentiate between the different heteromeric NR1-1a/NR2 combinations expressed in *Xenopus* oocytes (Yamakura et al., 1993).

Interestingly, NR1-1a/NR2A and NR1-1a/NR2A/NR2C receptors expressed in HEK 293 cells had similar affinities for \(^{3}H\) MK801 to that found for adult rat forebrain and mouse cerebellum membranes (Cik et al., 1993; Chazot et al., 1994). Thus providing evidence for the NMDA subunit complements present in the rat forebrain and cerebellum.
1.12.2.4 Modulation of heteromeric recombinant NMDA receptor function by polyamines, protons and ifenprodil

As discussed above (section 1.4.5), polyamines have a number of complex effects on the NMDA receptor function including two forms of potentiation which are dependent on the glycine concentration and a voltage-dependent block (Williams, 1997). The net effect of this complex interaction is concomitant with the NR2 subunit present in the heteromeric NMDA receptor complex (Williams et al., 1994). For example, glycine-independent spermine potentiation is critically dependent on the presence of the NR2B subunit, whereas glycine-dependent spermine potentiation is seen in NR1/NR2A, NR1/NR2B but not in NR1/NR2C and NR1/NR2D receptors (Zhang et al., 1994). The NR2 subunits also influence the pH sensitivity of the NMDA receptor with a profile that is different from that of spermine potentiation since only NR1/NR2C receptors exhibit reduced proton sensitivity (Traynelis et al., 1995). Thus different regions of the NR2 subunits are presumably involved in modulating responsiveness to spermine and protons.

Ifenprodil is a novel NMDA receptor antagonist that selectively inhibits NR1/NR2B receptors (Williams, 1993). In addition to its subtype-selectivity, an unusual feature of ifenprodil is a form of activity-dependent block since maximal inhibition by this ligand is seen at high concentrations of glutamate or NMDA. Secondly, ifenprodil has higher affinity for the agonist bound and desensitised states than for the closed or unbound states of the receptor (Kew et al., 1996). The mechanistic action of ifenprodil was
suggested to be via the polyamine binding site however it is now known that the polyamine and ifenprodil binding sites are distinct or interact via an allosteric mechanism (Kew and Kemp, 1998). More recently, site-directed mutagenesis experiments identified R337 in the NR2B subunit and D130 on the NR1 subunit as an absolute requirement for high affinity ifenprodil inhibition (Gallagher et al., 1996; Masuko et al., 1999). Mutations at these respective positions had little effect on the sensitivity to spermine and protons reinforcing the proposal that both ifenprodil and spermine actions are mediated through non-identical sites. However, some degree of overlap is suggested by other studies in which the substitution of aspartate D669 on the NR1-1a subunit abolishes or attenuates both glycine-dependent spermine potentiation and ifenprodil antagonism at NR1-1a/NR2B receptors (Kasiwagi et al., 1996).

1.12.2.5 Phosphorylation of heteromeric NMDA recombinant receptors

As with the NR1 subunit splice forms, (section 1.12.1) the type of NR2 subunit present in the heteromeric NR1/NR2 receptor complex appears to differentially regulate the phosphorylation of NMDA receptors. For example, PKC activation potentiates NR1/NR2A and NR1/NR2B receptors by increasing the opening probability of the channel and decreasing the affinity for Mg^{2+} ions, but has little effect upon NR1/NR2C and NR1/NR2D receptors (Chen and Huang, 1992). Recently, Grant et al., (1998) showed that the region sufficient to confer PKC phosphorylation effects require amino acids present in the carboxy domain (1300-1400) of the NR2A subunit. This was
deduced by constructing C-terminal NR2A/NR2C subunit chimaeras co-expressed with NR1-1a subunits in HEK 293 cells (Grant \textit{et al.}, 1998).

Tyrosine kinases can also potentiate glutamate-activated currents in HEK 293 cells co-expressing NR1/NR2A receptors. As with putative PKC phosphorylation sites, the C-terminal domain of the NR2A subunit has been suggested to contain the tyrosine phosphorylation sites (Y1105, Y1267 and Y1387) (Köhr and Seeburg, 1996). Furthermore, a possible mechanism for an activity-dependent feedback of the NMDA receptor has been suggested to be mediated via the binding of calmodulin-dependent kinase II (CaMKII) to NR1 subunits which causes a four-fold reduction in the open probability of heteromeric NR1/NR2 receptors (Ehlers \textit{et al.}, 1996).

\textbf{1.13 NON-NMDA RECEPTORS}

Activation of ionotropic non-NMDA receptors in the CNS displays fast neurotransmission at glutamatergic synapses that undergo marked desensitisation. Non-NMDA receptors are permeable to both Na$^+$ and K$^+$ ions. In contrast to NMDA receptors, both Ca$^{2+}$-impermeable and Ca$^{2+}$-permeable subtypes of the receptor exist (section 1.13.2) (Mayer and Westbrook, 1987; Iino \textit{et al.}, 1990). As stated above, the identification of selective ligands for non-NMDA receptors allowed these receptors to be differentiated into AMPA and kainate subtypes. However, it was unclear whether kainate receptors constituted an entirely separate group of proteins from AMPA receptors. For example, radioligand binding studies showed that kainate competes with
the radioligand binding of $[^3H]$ AMPA to adult cerebral cortical membranes (Honoré et al., 1982; Olsen et al., 1987). Additionally, functional studies demonstrated that kainate can behave as an agonist at receptors that display the pharmacological profile of AMPA receptors (Kiskin et al., 1986).

Many studies have now clarified that kainate receptors comprise a discrete population of receptors distinct from AMPA receptors. In support of this, autoradiographical studies within the rat vertebrate CNS using $[^3H]$ AMPA and $[^3H]$ kainate revealed a differential distribution of sites where $[^3H]$ kainate identified a high ($K_D = 5$ nM) and low ($K_D = 50$ nM) affinity binding sites (Foster and Fagg, 1984; Honoré et al., 1986).

Distribution of the high affinity kainate binding sites did not correlate with the high affinity $[^3H]$ AMPA radioligand binding sites (Young and Fagg, 1990).

1.13.1 Molecular biology of non-NMDA receptors

The first cDNA encoding a glutamate receptor, GluR1, from a rat cDNA library was identified by expression cloning (Hollmann et al., 1989). Subsequently, the sequence of GluR1 was used as a cDNA probe in homology screening and PCR to isolate additional related clones (reviewed in Hollmann and Heinemann, 1994). To date four AMPA receptor genes GluR1-4 (GluRA-D) cDNAs have been identified which encode protein polypeptides of 889, 862, 866 and 881 amino acids with predicted relative Mrs of 99, 96, 98 and 101 kDa respectively. Low-stringency hybridisation screening and PCR amplification with degenerate primers of AMPA receptor subunit clones identified
the first subunit cDNA of the kainate receptor, GluR5 (Bettler et al., 1990). In the following years additional members of the kainate family cDNAs were cloned including GluR6, GluR7, KA1 and KA2 (Egebjerg et al., 1991; Bettler et al., 1992). Kainate receptor subunits comprise of ~900 amino acids with a predicted molecular mass of ~100 kDa respectively.

The overall amino acid sequence identity between GluR1-GluR4 subunits is ~70%. These subunits are highly conserved between the rat, mouse and human species. In contrast to the AMPA receptor subunits, the kainate receptors are subdivided into two distinct classes based on amino acid homology and affinity for [³H] kainate. One group comprises GluR5-7 subunits that share 75% amino acid sequence identity with each other and exhibit low affinity for [³H] kainate. The second class includes the KA1 and KA2 subunits that display high affinity for [³H] kainate and are 68% homologous in amino acid sequence identity. Between the two groups i.e. GluR5-7 and KA1-2 the amino acid sequence identity is much lower at ~45% and is ~40% with the AMPA receptor subunits.

The proposed transmembrane topology of the non-NMDA receptor subunits is analogous to NMDA receptor subunits and is considered in section 1.7 and shown in Figure 1.6. Heterogeneity of the non-NMDA receptor subunit family is further increased by alternative splicing and RNA editing of the subunits. Each of the AMPA receptor subunits exists in one of two molecular forms which differ in the sequence of a segment of 38 amino acids found preceding the C-terminal transmembrane region due to alternative splicing (Sommer et al., 1990). The alternative exons are termed 'flip' and
'flop' isoforms (Figure 1.9). In addition, alternative splicing of the GluR4 subunit produces GluR4c owing to an alternative C-terminus of 38 amino acids (Gallo et al., 1992). Furthermore, alternative splicing of the GluR5 subunit yields two variants, GluR5-1 and GluR5-2 where the former contains an additional 15 amino acids in the N-terminal domain (Bettler et al., 1990). Carboxyl-terminus splice variants of the GluR5-2 subunit include isoforms GluR2a, b and c. The originally identified sequence is designated GluR5-2b while either an introduction of a stop codon produces a truncated form (GluR5-2a), or an in-frame insertion results in an elongated form (GluR5-2c) (Sommer et al., 1992). Two C-terminal alternative splice variants of the GluR7 subunit also exist termed GluR7-a and GluR7-b. GluR7-b differs from GluR7-a due to the addition of a 40 nucleotide cassette (Schiffer et al., 1997). As yet no splice variants of GluR6, KA1 and KA2 subunits have been reported.

Further heterogeneity of non-NMDA receptor subunits is introduced by the process of RNA editing which occurs at the Q/R site within the putative channel forming M2 region (Hume et al., 1991; Burnashev et al., 1992b). This mechanism of RNA editing occurs in the GluR2 which expresses an arginine codon (CIG) whereas the DNA sequence encoding subunits GluR1,3 and 4 all contain glutamine codon (CAG) in the homologous position within the M2 region (Sommer et al., 1991). Both the GluR5 and GluR6 are RNA edited at a homologous position to the GluR2, although the extent of editing is not 100% as found in the GluR2 subtype. GluR5 and GluR6 exist as approximately 39% and 75% in the edited (R) form in the adult brain. GluR6 can also undergo further editing at two more sites within the first hydrophobic domain where
isoleucine is replaced by valine and tyrosine by cysteine (Köhler et al., 1993). However, GluR7, KA1 and KA2 subunits do not undergo RNA editing as a Q residue is found at the homologous position within the M2 domain. Figure 1.10 shows the alignment of the amino acid residues at the Q/R site within the M2 region of ionotropic glutamate receptor subunits. The functional consequence of the amino acid change at the Q/R site is discussed below.

1.13.2 Pharmacological and functional properties of non-NMDA receptors

Clearly, the development of selective ligands for the AMPA and kainate receptors will aid in the discrimination between the different physiological roles of each type of receptor in vivo. However, due to the lack of selective AMPA or kainate receptor agonists meant that the AMPA receptors were initially defined pharmacologically by the rank order potency of agonists whereby, quisqualate > AMPA ~ domoate > L-glutamate > kainate. The rank order of agonist affinity at kainate receptors was domoate > kainate > L-glutamate >> AMPA.

More recently, a degree of pharmacological dissection between the AMPA and kainate receptors has been possible due to the development of a series of willardiine derivatives that behave as agonists at non-NMDA receptors. For example, radioligand binding studies showed that the halogenated willardiine derivative, $[^3H]$(s)-5-fluorowillardiine displayed a selectivity of ~ 46-fold for AMPA receptors compared with kainate receptors (Wong et al., 1994; Hawkins et al., 1995) whereas (s)-5-iodowillardiine
displayed ~130-fold selectivity for kainate receptors in the dorsal root ganglion (DRG) (Wong et al., 1994). More recently, a potent and highly selective kainate receptor agonist has been described, (2S,4R)-4-methylglutamic acid (SYM2081), which possesses nanomolar potency and ~3000-200-fold selectivity for kainate versus AMPA and NMDA receptors respectively (Zhou et al., 1997).

The desensitisation properties of the non-NMDA ionotropic receptors to agonists such as cyclothiazide has allowed a further degree of differentiation between non-NMDA receptors since cyclothiazide, facilitates agonist responses at the AMPA receptor by specifically inhibiting desensitisation of the receptor but it has no effect on kainate receptors (Partin et al., 1993). In contrast, a range of plant lectins including Concanavalin A (Con A) prevents desensitisation of kainate receptors with little effect on AMPA receptors (Huettner, 1990; Partin et al., 1993).

To date, competitive antagonists that are currently available include 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-cyano-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX). The former exhibits a 5-fold and the latter compound displays a 30-fold selectivity for AMPA receptors in radioligand binding studies (Honoré et al., 1988; Sheardown et al., 1990). In contrast, functional studies show that the selectivity of these two antagonists is very modest since CNQX possesses minimal selectivity and NBQX displayed only a weak 3-fold selectivity between AMPA and kainate receptors in the DRG (Lodge et al., 1991). The first reported selective kainate receptor antagonist was 5-nitro-6,7,8,9, tetrahydrobenzo[G]indole-2,3-dione-3-oxime (NS-102) which displayed a 20-fold selectivity for kainate versus AMPA receptor-mediated responses (Wilding
and Huettner, 1996). However, some reports claim that NS-102 exhibits a similar affinity for both AMPA and kainate receptors (Paternain et al., 1996). These contradicting reports about the selectivity of NS-102 have been a pivotal factor in the demise of this compound. A recently developed non-competitive antagonist has shown a promising selectivity profile; 1-(4-aminophenyl)-3-methyl-carbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydroxy-5H-2,3-benzodiazepine (GYKI53655) (Wilding and Huettner, 1995). This compound has been described as a selective AMPA receptor antagonist (Bleakman et al., 1996). Competition radioligand binding studies on native AMPA receptors show that the action of [3H] GYKI53655 is mediated at a site distinct from the binding sites of competitive agonists and antagonists (Wilding and Huettner, 1995).

Future studies concentrated efforts away from native systems but employed recombinant expression systems to develop subtype-selective ligands for the AMPA and kainate receptors. Selective kainate receptor ligands developed include the agonist, (RS)-2-amino-3(3-hydroxy-5-tert-butylisoaxal4-yl)propanoic acid (ATPA) which expresses selectivity for GluR5 subunits compared with AMPA receptor subunits, GluR7 and KA2 with no appreciable activity at GluR6 (Clarke et al., 1997). Furthermore, the competitive kainate receptor antagonist, (3S,4aR,6R,8aR)-6-[2-(1(2)H-tetrazol-5-yl)methyl]oxymethyl)-1,2,3,4,4a,5,6,7,8,8adecahydroisoquinoline-3-carboxylic acid (LY294486) selectively inhibits [3H] kainate radioligand binding to homomeric GluR5 receptors with little effect on GluR6, GluR7, KA2 and AMPA receptors (Clarke et al., 1997).
As with NMDA receptors, the functional and pharmacological properties of non-NMDA receptor subunits using both Xenopus oocytes and mammalian cell expression systems have been characterised. Analogous to the NR1-1a subunit, non-NMDA receptor subunits GluR1-4 and GluR5-7 can all form functional homomeric receptor channels following expression in Xenopus oocytes (Hollmann et al., 1989; Hollmann and Heinemann, 1994; Bettler and Mulle, 1995; Schiffer et al., 1997). The rank order of agonist potency for homomeric AMPA receptors from electrophysiological and [³H]AMPA radioligand binding studies was quisqualate > domoate ~ AMPA > L-glutamate > kainate (Hollmann et al., 1989; Nakanishi et al., 1990; Sakimura et al., 1990). Homomeric kainate receptors displayed a rank order of agonist potency of domoate > kainate >> L-glutamate (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). In contrast, both KA1 and KA2 subunits do not form functional homomeric channels (Hollmann and Heinemann, 1994; Bettler and Mulle, 1995). However, radioligand binding studies showed that KA1 and KA2 subunits expressed in mammalian cells can bind to non-NMDA receptor ligands where the order of agonist binding affinity was [³H]kainate > [³H]quisqualate > [³H]domoate > [³H]L-glutamate (Werner et al., 1991; Herb et al., 1992).

Expression of homomeric channels composed of GluR1, GluR3 or GluR4 subunits in Xenopus oocytes or HEK 293 cells displayed strong inward rectification which was Ca²⁺-permeable (Hollmann et al., 1991; Verdoorn et al., 1991; Dingledine et al., 1992). In contrast, GluR2 channels showed linear current/voltage (I/V) relationships and were Ca²⁺-impermeable (Hollmann et al., 1991; Verdoorn et al., 1991). The difference in the
Ca²⁺ ion permeability of the GluR2 compared with GluR1,3 and 4 channels is due to the amino acid change at the Q/R RNA edited site in the putative M2 domain (section 1.13.1). As discussed above, GluR1,3 and 4 subunits contain a Q residue that is replaced by a positively charged R residue in the homologous position in the GluR2 subunit. Similarly, edited homomeric GluR5 and GluR6 channels i.e. channels that contain an R residue at the Q/R site display linear or slightly outwardly rectifying I/V relationship and have significantly reduced Ca²⁺-permeability compared with the unedited forms (Egebjerg and Heinemann, 1993). Site-directed mutagenesis experiments have further verified that these disparate channel properties between the non-NMDA receptor subunits are determined by the Q/R site. These studies have shown that replacing the Q residue in GluR1 subunit at the Q/R site for an R residue dramatically changes the channels properties to those observed in GluR2 subunits i.e. Ca²⁺-impermeable with linear I/V characteristics (reviewed in Hollmann and Heinemann, 1994). On the basis of these findings it has been proposed that the ring of positively charged arginine residues within the putative pore-lining hydrophobic domain constitute an energy barrier to the movement of divalent cations such as Ca²⁺ ions (Burnashev et al., 1995).

The co-expression of GluR1 with GluR3 or GluR4 produces heteromeric channels that have gating and ion permeability properties similar to their respective homomeric channels i.e. outwardly rectifying I/V relationship and reduced Ca²⁺-permeability. However, co-assembly with a GluR2 forms channels that are Ca²⁺-permeable hence demonstrating the dominance of the GluR2 subunit in gating and ion permeability. The introduction of edited kainate subunits into receptors consisting of unedited subunits
produces heteromeric channels with similar functional properties to those observed in homomeric edited complexes. Co-expression of GluR1-4 with GluR5-7 subunits does not generate responses that are different from the respective homomeric complexes indicating that interaction between these receptor subunits is unlikely (reviewed in Hollmann and Heinemann, 1994).

Furthermore, alternative splicing of non-NMDA receptors confers distinct channel properties such that those receptors containing flip modules appear to be characterised by slower desensitisation kinetics than receptors that contain the flop splice variant (Sommer et al., 1990).

1.14 METABOTROPIC GLUTAMATE RECEPTORS

The mGluRs were identified in the mid 1980s when evidence began to emerge of glutamate stimulation of phospholipase C (PLC) and phosphoinositide hydrolysis (Sladeczek et al., 1985). In subsequent years, it was demonstrated that these receptors can couple either directly or indirectly to a range of second messenger systems (Prezeau et al., 1992) suggesting that a family of mGluR subtypes exists. Both molecular biological and pharmacological tools have aided in the characterisation of mGluRs.
1.14.1 Molecular biology of metabotropic glutamate receptors

The first cDNA encoding a mGluR subunit was cloned independently by two groups using expression cloning in *Xenopus* oocytes and it was termed mGluR1 (Houamed *et al.*, 1991; Masu *et al.*, 1991). In the following years, low stringency hybridisation or PCR using degenerate primers of the mGluR1 sequence isolated a further seven mGluR subtypes, i.e. mGluR1-8 (Suzdaz *et al.*, 1994). These identified subtypes can exist as multiple splice variants which are shown in Table 1.1. The primary sequence predicts the structure of the mGluR to have an unusually large extracellular N-terminal domain followed by seven transmembrane spanning regions with the carboxyl terminus sited on the intracellular side of the plasma membrane (Figure 1.11). The mGluRs are larger in size and have low amino acid sequence homology compared to previously identified G-protein coupled receptors (GPCRs). On the basis of sequence homology, agonist selectivity and second messenger-coupling as discussed below, the mGluR family has been subdivided into three groups. Group I comprises mGluR1a-e and 5a-b; Group II includes mGluR2 and 3; while Group III contains the remaining mGluR subunits 4a-b, 6, 7 and 8 (Table 1.1). Sequence analysis algorithms searching for identity between the mGluR subunit family revealed that within the same group, the subtypes exhibited ~70% amino acid sequence homology whilst between groups it falls to ~45%.
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1.14.2 Pharmacological characterisation of metabotropic glutamate receptors

The direct correlation of glutamate action coupled to PLC via G-proteins was demonstrated by injecting Xenopus oocytes with rat brain mRNA. Only glutamate, quisqualate and ibotenate but not NMDA or AMPA could elicit responses (Sugiyama et al., 1987). Hence, this study identified quisqualate and ibotenate as agonists acting at the mGluR. However, both these ligands cross react at ionotropic glutamate receptors consequently, future studies directed research into the development of selective ligands for the mGluRs. Specific receptor agonists identified include trans 1-aminocyclopentane-1,3-dicarboxylate (ACPD) and L-2-amino-4-phosphonobutyrate (L-AP4). In addition to these agonists, antagonists for mGluR were also developed including α-methyl-4-carboxyphenylglycine (MCPG) and L-2-amino-3-phosphopropionic acid (L-AP3) (reviewed in Pin and Duvoisin, 1995).

Expression of recombinant mGluRs in heterologous systems has allowed the subdivision of the mGluR subunit family as stated above based on their second messenger transduction mechanisms and agonist selectivity (Table 1.1) (Abe et al., 1992; Aramori and Nakanishi, 1992; Tanabe et al., 1992). For example, Group I mGluRs are linked to PLC and thus phosphoinositide hydrolysis and Ca\(^{2+}\) mobilisation (Abe et al., 1992). Quisqualate represents the most potent agonist at this group (Aramori and Nakanishi, 1992). However, a recently synthesised agonist, (Z)-amino-3-[2'-(3',5'-dioxo-1',2',4'-oxadiazolidiny1)]cyclobutane-1-carboxylic acid (Z-CBQA) has been described which allows differentiation between the mGluR subtypes that comprise
Group I. This agonist, Z-CBQA, displays 10-fold higher affinity mGluR5 in comparison to mGluR1 receptors (Littman et al., 1999). In contrast, Groups II and III are negatively coupled through adenyl cyclase to cyclic 3',5'-adenosine monophosphate (cAMP) formation. Groups II and III are characterised by their distinct agonist selectivity; Group II is activated most potently by the agonist, (+)-2-aminobicyclo-[3.1.0]-hexane-2,6-dicarboxylate (LY354740). This compound has an affinity in the nanomolar range for these receptors with a modest 5-fold selectivity for mGluR2 over mGluR3 subtypes (Malherbe, 1999). While in Group III; L-AP4 is the most selective agonist nonetheless, within Group III the agonist, (R,S)-4-phosphononophenylglycine ((RS)-PPG) shows ~20-fold selectivity for mGluR8 over mGluR4 or mGluR6 receptors (Gasparini et al., 1999).

The development of antagonists for mGluRs has been complex since some compounds act as antagonists at one receptor subtype but agonists at another mGluR. Nevertheless, to date the most potent and selective antagonists at mGluRs have been derived from phenylglycine. The phenylglycine derivatives, (s)-4-carboxy-3-hydroxyphenylglycine (4C3HPG), (s)-3-carboxy-4-hydroxyphenylglycine (3C4HPG), (s)-4-carboxyphenylglycine (4CPG) and (+)-MCPG all effectively antagonised the action of L-glutamate on mGluR1 (Hayashi et al., 1994; Watkins and Collingridge, 1994). However, (s)-4C3HPG, (s)-3C4HPG and (s)-4CPG, also displayed agonist activity at mGluR2 and (+)-MCPG proved to be an effective antagonist of mGluR2 subunits (Hayashi et al., 1994; Watkins and Collingridge, 1994). To date the most potent Group II mGluR antagonist is 2-amino-2-(2-carboxycyclopropan-1-yl)-3-(dibenzopyran-4-yl)
propanoic acid (LY341495), which has nanomolar affinity at this subclass of mGluRs (Cartmell et al., 1998). Until recently, very few subtype-selective antagonists were available for the Group III mGluRs, (S)-2-amino-2-methyl-4-phosphonobutanoic acid/alpha-methyl (MAP-4) and (R,S)-alpha-methylserine-O-phosphate (MSOP) have been described as the most potent and selective antagonists of this group. Although, some reports claim that MAP-4 may possess agonist activity at mGluR4 and mGluR6 receptors (Laurie et al., 1997). These recently identified antagonists could lead the development of further subtype-selective ligands to better understand the physiological roles of the mGluR subtypes in the CNS.

1.15 PHYSIOLOGICAL ROLES OF GLUTAMATE RECEPTORS

1.15.1 Transgenic studies

Extrapolation of the disparate NMDA receptor subunit functional roles in the CNS has been achieved by transgenic technology. For example, deletion of the NR1 gene was found to be a lethal mutation which resulted in mutant mice dying within 10-20 h perinatally (Forrest et al., 1994) thereby confirming that the NR1 subunit is an obligatory subunit of the NMDA receptor. In contrast, mice lacking the NR2A, NR2C or NR2D subunit were all viable (Ikeda et al., 1995; Sakimura et al., 1995; Ebralidze et al., 1996). However, NR2A subunit-lacking mice performed poorly in spatial learning tasks and long term potentiation (LTP) (section 1.15.2) was reduced but not abolished.
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(Sakimura et al., 1995). Both NR2C and NR2D gene ‘knockout mice’ displayed no detectable deficits (Ikeda et al., 1995; Ebralidze et al., 1996). Mutant mice lacking the NR2B subunit have shown a complete loss of NMDA receptor-mediated responses in the hippocampus (Kutswada et al., 1996). This mutation also effected both long term depression (LTD) (section 1.15.3) and the formation of neuronal patterns in the trigeminal nucleus (Kutswada et al., 1996). Thus these correlated observations suggest that the NR2B subunit is important in synaptic plasticity and neuronal formation.

1.15.2 Long term Potentiation

As discussed above, excitatory amino acids receptor activation appears to be important in both the formation of synaptic connections during development and in determining the strength of synaptic contacts. LTP is an example of an experimental paradigm that represent an increase in synaptic transmission following an concomitant stimulation of both pre- and post-synaptic excitatory neurons in the CA1 region of the hippocampus (Anwyl, 1989; Bliss and Collingridge, 1993). However, this effect has been also reported in all excitatory pathways in the hippocampus, amygdala and cortex (Clugnet and LeDoux, 1990). The physiological importance of the induction of synaptic strengthening lies in learning and memory (Bliss and Lomo, 1973). The characteristic properties for the activation of the NMDA receptor including its requirement of both pre-synaptic activity to release L-glutamate and post-synaptic depolarisation to alleviate the Mg$^{2+}$ block of the channel are consonant with it being involved in the induction of
LTP. Furthermore, systemic administration of an NMDA receptor antagonist, APV, was shown to inhibit the induction of LTP in the hippocampus (Collingridge et al., 1983). In addition, transgenic studies have demonstrated the importance of NMDA receptor subunits in learning as discussed in section 1.15.1.

Experimental observations have suggested that Ca\(^{2+}\) ions are required for LTP propagation since Ca\(^{2+}\) chelators have been shown to prevent the induction of LTP (Lynch et al., 1983). However, it remains unclear whether the Ca\(^{2+}\) signal is amplified by the release of intracellular Ca\(^{2+}\) stores or whether the Ca\(^{2+}\) influx via the NMDA receptor alone is sufficient to induce LTP (Svoboda and Mainen, 1999). The biochemical pathways activated by the increase of Ca\(^{2+}\) levels also remain a matter of debate but a number of candidates have been proposed based on experimental observations. Multiple reports indicate CaMKII as a pivotal molecular component of LTP since genetic deletion of CaMKII prevents the induction of LTP (Malenka et al., 1989). However, research is ongoing to identify the Ca\(^{2+}\)-activated signal transduction mechanisms that lead to the sequence of events that culminate in enhanced synaptic transmission.

Recent evidence has also indicated a potential function of mGluRs in LTP. For example, application of a mGluR agonist, ACPD, in the hippocampus has been reported to induce LTP (Ben-Ari et al., 1992). The role of mGluR in LTP however has been suggested to be short lived as the initial stimulation of mGluRs presumably activates a phosphorylation event which then negates for subsequent mGluR activation during the propagation of LTP (Bortolotto et al., 1994).
1.15.3 Long term depression

In contrast to LTP, LTD is the term that refers to a sustained reduction in AMPA receptor-mediated activity at the parallel fibre-purkinje neuronal synapses that are active during climbing fiber stimulation (Ito et al., 1982; Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). Purkinje cells receive two major excitatory inputs from both climbing fibres that synapse directly with one purkinje neuron and those from parallel fibres which synapse with many purkinje neurons. It is well accepted that the induction of LTD requires the simultaneous activation of both parallel and climbing fibres (reviewed in Linden and Connor, 1993). The physiological importance of LTD has been proposed to provide a cellular mechanism in the function of cerebellar purkinje cells that modulate sensory information that in turn control voluntary movements and reflexes. The induction of LTD is thought to be mediated by the rise of both intracellular Na$^+$ and Ca$^{2+}$ ions via both AMPA receptors and stimulation of PLC through mGluRs. The evidence in support of this model for the induction of LTD is universally agreed upon. For example, both specific mGluR and AMPA receptor antagonists completely block the generation of LTD (Linden et al., 1991). Similarly, replacement of Na$^+$ with Li$^+$ or Cs$^+$ ions prevents the occurrence of LTD thus suggesting that Na$^+$ influx via AMPA receptor channels is an important criterion for the propagation of LTD (Linden and Connor, 1993). Analogous to LTP, the role of Ca$^{2+}$ ions in the induction of LTD was supported by the finding that the induction of LTD in purkinje neurons was occluded by Ca$^{2+}$ chelators (Sakurai, 1990).
The downstream signal transduction mechanisms that are activated by the increase of intracellular Ca\(^{2+}\) and Na\(^{+}\) ions are unclear. However, PKC activation has been identified as a key component for the induction of LTD although it remains ambiguous whether PKC activation is required for continual LTD expression (Crepel and Krupa, 1988). Furthermore, it has recently been shown that nitric oxide (NO) is released after climbing fibre stimulation and that LTD is blocked by haemoglobin which strongly binds NO (Shibuki and Okada, 1991). In addition, exogenous NO or cyclic guanidino monophosphate (cGMP) can substitute for the stimulation of climbing fibres to cause LTD. These observations highlight that the NO/cGMP cascade may also have a role in the induction of LTD (reviewed in Linden and Conner, 1993).

Both LTP and LTD demonstrate how different glutamate receptors determine the formation and efficacy of synaptic transmission. However, overactivation of excitatory amino acids receptors can lead to acute neuronal death in response to a variety of insults to the CNS as discussed below (Choi, 1988).

1.15.4 Glutamate excitotoxicity and therapeutic intervention by NMDA receptor antagonists

Knowledge that glutamate is potentially toxic to central neurons dates back as early as 1957 when Lucas and Newhouse found that high doses of glutamate administrated systemically induced neurodegeneration. In the following years, Olney and co-workers demonstrated that systemic administration of glutamate to brain regions caused post-
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synaptic neuronal cytotoxicity (Olney, 1990). Over the years there has been a wealth of reports similar to these describing the detrimental effects of glutamate as a mediator of excitotoxic cell death.

More recently, substantial evidence has accumulated implicating excitotoxicity in the pathogenesis of a number of neurodegenerative disorders including ischaemic brain injury such as stroke (Rothman and Olney, 1986; Choi, 1988). Most strokes are caused by an interruption of arterial blood flow by thrombosis to the brain leading to cerebral ischaemia. The sequence of events that induce the ischaemic cascade begins with hypoxia i.e. a lack of oxygen causing a spread of perinfarct depolarisations leading to the synaptic release of glutamate. This resultant glutamate build-up causes the overactivation of ionotropic glutamate receptors and consequently a large influx of both Ca\(^{2+}\) and Na\(^+\) ions. The mechanisms of neuronal death associated with these ionic imbalances are mediated by two major pathways. Firstly, the large influx of Na\(^+\) ions is accompanied by an influx of both Cl\(^-\) and H\(_2\)O leading to acute cell swelling. Secondly, the excessive Ca\(^{2+}\) ion influx through glutamate receptors causes the activation of proteases, lipases, impaired mitochondrial function and the generation of free radicals via the arachidonic acid or the NO synthase (NOS) cascades. The effects of these downstream pathways probably culminates to events leading to cell death.

The direct correlation between excessive NMDA receptor activation and cell death arises from reports showing that exposure to cultured hippocampal slices to NMDA for only 15-30 min was sufficient to cause neuronal death (Rothman, 1985). Furthermore, NMDA receptor antagonists could attenuate cultured neuronal cell death induced by
INTRODUCTION

oxygen and glucose deprivation (Choi, 1988). Besides *in vitro* studies, *in vivo* investigations have also demonstrated that NMDA receptor antagonism reduces infarct volume in animal models of focal ischemia (Albers *et al*., 1992).

Therefore several paths of research began to investigate the targeting of the NMDA receptor to stem the excessive Ca\(^{2+}\) influx associated with this receptor to reduce ischaemic damage. A number of key regulatory sites on the NMDA receptor complex including the ion channel pore and glutamate and glycine binding sites were identified for the development of NMDA receptor antagonists. For example, the development of the channel blocker, MK801, and the glutamate antagonist, Selfotol, proved to be efficacious in the middle cerebral artery occlusion (MCAo) animal model of stroke (Bullock *et al*., 1990; Scatton, 1994). However, both these compounds displayed several pre-clinical side effects including psychomimetic effects, neuronal vacuolisation and degeneration in limbic regions of the neocortex of rodents (Ellison, 1994; Grotta *et al*., 1995). In contrast, glycine site antagonists exhibited reduced side-effect profiles compared with those seen with non-competitive channel blockers and glutamate site antagonists. This promising side effect profile of glycine site antagonists could be possibly explained by the mechanism of action of these drugs. In the presence of low concentrations of glycine it has been shown that the binding of glutamate to the NMDA receptor initiates a rapidly desensitising response. However, during an ischaemic insult the extracellular glycine concentration is increased (Globus *et al*., 1991) thus causing a reduction in the level of NMDA receptor desensitisation. Therefore, under excitotoxic conditions, glycine site antagonists may cause a concomitant increase in NMDA
receptor desensitisation which could result in a greater separation between anti-
excitotoxic and unwanted side effects (Parsons et al., 1993).

In recent years as previously described (section 1.4.2), a number of glycine site
antagonists have been developed which include ACEA 1011, 5,7 DCKA, L689,560,
MDL105,519, GV150,526A and GV196,771A (reviewed in Danysz and Parsons, 1998).
Only GV150,526A and GV196,771A progressed into clinical trials. Previous studies
have shown that GV150,526A is a selective NMDA receptor glycine site antagonist
which reduces the infarct area and protects somatosensory evoked potentials in the
MCAo model of focal ischaemia in the rat (Bordi et al., 1997). It is devoid of amnestic
side effects at doses well above the neuroprotective range of action (Bordi et al., 1996).

However in 2000, GV150,526A was withdrawn from Phase III clinical trials for the
treatment of stroke and traumatic brain injury as it was found to bind to the plasma
binding protein, bilirubin. In contrast to GV150,526A, GV196,771A's in vivo properties
include a reduction in the pain sensitivity in the second phase of inflammation in the
formalin test (Bennet and Xie, 1998) and it also blocks the sensitisation caused after
chronic constriction injury of the left sciatic nerve in rat (Quartaroli et al., 1999). It was
hoped that GV196,771A would be an anti-hyperalgesic compound useful in the
treatment and prevention of chronic pain. However, this compound was suspended from
Phase IIb clinical trials and it is currently being employed as a template in the
development of the next generation of glycine site antagonists. As yet, no information
explaining the demise of GV196,771A from clinical trials is available.

As discussed above to date most of the clinical results using NMDA receptor
antagonists as potential therapeutic targets for the treatment of stroke have been disappointing. There are several reasons why NMDA receptor antagonists have performed poorly in clinical trials. For example, these compounds have been assessed in stroke-induced animal models. Pharmacokinetics and the severity of an insult between animals and humans may be different. Consequently, neuroprotective effects of NMDA receptor antagonists in pre-clinical stroke models may not extrapolate into effective clinical compounds.

An alternative explanation questions whether NMDA receptor antagonism is the predominant mechanism for neuroprotection. The ischaemic environment in the brain consists of high concentrations of protons, free radicals and zinc (Aizenman et al., 1989; Tang et al., 1990). This type of micro-environment is consonant with the attenuation of NMDA receptor function. Under these conditions, it is probable that an increase in the activation of AMPA and kainate receptors in glutamate-induced excitotoxicity could be potentially predicted (McDonald, 1998). Consistent with the latter proposal studies have implicated non-NMDA receptors as the main mediators of neurotoxicity (Mattson et al., 1989; Frandsen et al., 1990). Moreover, non-NMDA receptor antagonists were also effective in preventing neuronal damage in models of global ischemia (Sheardown et al., 1990). Accordingly, the new line of momentum for neuroprotection stems towards the dual inhibition of both NMDA and AMPA/kainate receptors in order to increase the overall anti-excitotoxic efficacy on ischaemia neurons.

Traditional lines of thought implicating excitatory-triggered Ca\(^{2+}\) overload in mechanisms of ischaemic brain injury have also been challenged. The Ca\(^{2+}\) homeostasis
in cells seems to be of paramount importance to the mode of cell death undertaken i.e. necrosis or apoptosis. In some cases, apoptosis can be attenuated following inhibition of calcium sequestration (Lampe et al., 1995). Survival of neuronal cells therefore seems to be dependent on the intracellular Ca\(^{2+}\) levels. NMDA antagonist application as a therapy of ischaemic injury may have a beneficial role in attenuating excitotoxicity, but also could cause calcium starvation in neurons leading to apoptosis. In support of this finding, studies have shown that NMDA antagonists can enhance neuronal apoptosis (Koh and Cotman, 1992).

These correlated observations suggest that future therapeutic directions require a refinement of the glutamate receptor antagonist therapy. Currently, there has been a trend aimed in development of agents blocking the downstream effects of calcium ions. For example, inhibition of proteases such as calpain (reviewed in Lee et al., 1999).

1.16 THE OBJECTIVES OF THE STUDY

The focus of this thesis was to investigate the NMDA receptor subtype-selectivity of two novel glycine site antagonists, GV150,526A and GV196,771A by assaying their ability to inhibit the radioligand binding of \(^{3}H\) MDL105,519, to both native NMDA receptors and HEK 293 cells transfected with various combinations of NMDA receptor clones. The competition of \(^{3}H\) MDL105,519 radioligand binding to heteromeric NMDA receptors by both GV150,526A and GV196,771A yielded interestingly, biphasic displacement curves. Consequently, many approaches were used to characterise the
multiple binding sites resolved including studying the competition profiles for the inhibition \( ^{3}\text{H} \) MDL105,519 radioligand binding to NR1-1a/NR2A receptors by a series of glycine site ligands with diverse chemical structures, allosteric influences of glutamate on the affinities of both GV150,526A and GV196,771A for NR1-1a/NR2A receptors and intact cell surface radioligand binding to investigate the displacement by both GV150,526A and GV196,771A of \( ^{3}\text{H} \) MDL105,519 radioligand binding to cell surface receptors versus total receptor populations.
Figure 1.0: Structure of excitatory amino acid receptor agonists

- Glutamate
- Aspartate
- NMDA
- Quisqualate
- Kainate
- AMPA
Figure 1.1: A phylogenetic tree of ionotropic glutamate receptor subunits

Schematic tree diagram showing clusters of relationships among the ionotropic glutamate receptor subunit families. The classification is based on the comparison of the predicted amino acid sequence identity between the ionotropic glutamate receptor subunits.
The NMDA receptor requires the binding of both glutamate and the co-agonist glycine and the relief of a voltage-dependent extracellular Mg\(^{2+}\) block for receptor activation (Mayer et al., 1984; Johnson and Ascher, 1987). The channel is primarily permeable to K\(^+\), Na\(^+\) and Ca\(^{2+}\) ions (Mayer and Westbrook, 1987). Functional NMDA receptors are composed of NR1 and NR2 subunits where the former subunit contains the glycine binding site and the glutamate recognition site is located on the NR2 subunit (Kuryatov et al., 1994; Laube et al., 1997). The major modulatory target sites are shown including that for polyamines which are positive modulators of the channel but they can also block the channel at higher concentrations (Williams et al., 1993). Ifenprodil is a selective antagonist for NR2B-containing receptors (Williams et al., 1993). The open NMDA channel is blocked by non-competitive antagonists such as MK801 in a voltage-dependent manner (Wong et al., 1986). In addition to the extracellular Mg\(^{2+}\) block site there is also an internal Mg\(^{2+}\) block site situated within the receptor channel (Nowak et al., 1984; Johnson and Ascher, 1990). The diagram is not to scale.
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NR1-1a NR2B

Na⁺ Ca²⁺

M₁ M₃ M₂

C₂

COOH

Modulators

Channel blockers

Polyamines

MK801

Ifenprodil

Internal Mg²⁺ Block

External Mg²⁺ Block

-67-
Figure 1.3: Structure of the NMDA receptor glycine site antagonists

- 5,7 DCKA
- GV150,526A
- L701,324
- GV196,771A
- MDL105,519
- L689,560
Figure 1.4: Schematic representation of the NMDA receptor subunits

The diagram shows the six subunits of the NMDA receptor. The putative transmembrane regions are indicated by filled boxes. The number of amino acids residues of the proposed mature rat subunits are shown by the scale.
Figure 1.4: Schematic representation of the NMDA receptor subunits

The diagram shows the six subunits of the NMDA receptor. The putative transmembrane regions are indicated by filled boxes. The number of amino acids residues of the proposed mature rat subunits are shown by the scale.
Figure 1.6: The proposed transmembrane topology of NMDA receptors with inclusion of agonist binding sites

Proposed transmembrane topology of the NMDA receptor subunits is shown which is analogous with other members of the AMPA and kainate receptor subunit families (Hollmann et al., 1994). S1 and S2 regions are predicted to form the binding domain for glycine (or glutamate for NR2 and non-NMDA receptor subunits). A, being the original model with four transmembrane regions (M1-M4) where the topological organisation of the amino-terminus and the carboxyl-terminus are placed on extracellular membrane surface (Hollmann et al., 1989). B, is the current model where the features include three transmembrane spanning domains (M1, M3 and M4) and the putative M2 segment is thought to form a hairpin turn with the membrane (Hollmann et al., 1994; Wo and Oswald, 1994; Hirai et al., 1996). The diagram is not to scale.
Putative transmembrane domains and the monotopic M2 region are shown by the black boxes, the amino terminal domain is shown by a grey box and the hatched box at the amino terminus represents a signal peptide of ionotropic receptors. Detailed amino acid sequence comparisons followed by multiple alignments of the ionotropic glutamate receptor subunit families with various PBPs led Nakanishi et al., (1990) and O'Hara et al., (1993) to recognise that several regions of amino acid sequence similarities exist between these protein families. It was proposed that the first 400 amino acids of ionotropc glutamate receptors share a common folding motif with the LIVBP shown by the grey box. S1 and S2 domains of ionotropic receptors are homologous in amino acid sequence identity to various PBPs such as QBP and LOABP. PBPs are denoted by unfilled boxes. Modified after Pass, (1988).
Figure 1.8: Glycine binding site of the NR1 subunit modelled on the bilobular 3D structure of LOABP

The NR1 substitutions affecting glycine affinity revealed by Hirai et al., (1996) (●) and Kuryatov et al., (1994) (○) are projected schematically onto the three-dimensional structure of LOABP (Oh et al., 1993). In this model, the transmembrane segments M1-M3 are replacing the hinge region of LOABP, which connects lobes 1 and 2. The putative hinge region 2 indicated by a dotted line contains residues F735 and F736 identified as the major determinants of glycine binding. The amino acid sequences connecting lobes 1 and 2 to transmembrane segments as well as the (−) and C-terminal extensions of the NR1 subunit, are not represented to scale. The M2 region is drawn as a re-entrant loop. After Hirai et al., (1996).
Figure 1.9: Proposed secondary structure of AMPA and kainate receptor subunits

All ionotropic non-NMDA glutamate receptor subunits share a common transmembrane topology. Features of the predicted protein structure of these subunits include three transmembrane regions, (M1, M3 and M4) and one domain forming a loop within the membrane (M2) leading to a C-terminus located intracellular (Wo and Oswald, 1995). Receptor subunit heterogeneity occurs additionally by the process of RNA editing and alternative splicing. RNA editing at M1 is exclusive to the GluR6 subunit which results in a change of amino acid, I to V. Further editing in this region leads to a C while unedited RNA encodes for a Y residue. RNA editing also occurs at the M2 domain in the AMPA subunit GluR2 and kainate subunits GluR5 and 6. This position is referred to as the Q/R site where the edited subunits contain an R residue and the unedited subunits contain a Q residue. AMPA receptor subunits exist in two major forms with
respect to an alternatively spliced exon sequence of 38 residues (Sommer et al., 1990). The two forms are named 'flip' and 'flop' and are located on the extracellular surface preceding the M4 region (modified from Seeburg, 1993). The diagram is not to scale.
### Predicted membrane-spanning region

<table>
<thead>
<tr>
<th></th>
<th>GluR1</th>
<th>GluR2</th>
<th>GluR5</th>
<th>GluR6</th>
<th>KA1</th>
<th>KA2</th>
<th>NR1</th>
</tr>
</thead>
</table>

**Figure 1.10:** Alignment of amino acid sequences in the putative channel forming region of ionotropic glutamate receptor subunits

Polypeptide sequences in the M2 region of the ionotropic glutamate receptor subunits are shown in the boxed area. The crucial position controlling ion flux (Q/R/N site) is shown in grey. The two amino acid residues at this position in GluR5 and GluR6 subunits indicate the existence of edited/non-edited forms. After Sommer et al., (1992).
Schematic representation of the secondary structure of metabotropic glutamate receptor subunits. The primary sequence predicts the structure of the mGluR subunit to have an unusually large extracellular N-terminal domain, followed by seven transmembrane spanning regions (M1-M7), with the carboxyl-terminus situated on the intracellular side of the plasma membrane. The diagram is not to scale.

Figure 1.11: Schematic representation of the putative secondary structure of metabotropic glutamate receptor subunits
Table 1.1: Summary of the classification of the metabotropic glutamate receptor family

The metabotropic glutamate receptor subunits are subdivided into three groups based on their amino acid sequence homology, agonist selectivity and signal transduction mechanisms.

<table>
<thead>
<tr>
<th>Group</th>
<th>Selective agonist</th>
<th>Signal transduction mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR1a</td>
<td>Quisqualate</td>
<td>PLC activation</td>
</tr>
<tr>
<td>mGluR1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR1d</td>
<td></td>
<td></td>
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<tr>
<td>mGluR1e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR5a</td>
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<td></td>
</tr>
<tr>
<td>mGluR5b</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR2</td>
<td>LY354740</td>
<td>Negatively coupled to adenylate cyclase</td>
</tr>
<tr>
<td>mGluR3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR4a</td>
<td>L-AP4</td>
<td>Negatively coupled to adenylate cyclase</td>
</tr>
<tr>
<td>mGluR4b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGluR6</td>
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<td></td>
</tr>
<tr>
<td>mGluR7</td>
<td></td>
<td></td>
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<tr>
<td>mGluR8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS

Bacterial culture media components (bactotryptone, bactoyeast extract and bacto-agar) were from Difco Labs., (East Mosely, U.K). Bacterial *E. coli* strain used was INV1α (DH1 derivative, genotype; endA1, recA1, hsdR17(R<sup>K,M</sup>), supE44, λ<sup>+</sup>, thi-1, gyrA, relA, Φ80 lacZΔM15Δ, (lacZYA-argF), deoR<sup>+</sup>, F) (D2) and it was obtained from British Biotechnology Ltd. (Oxford, UK). Ampicillin, DNA molecular weight markers II (λ DNA, Hind III digested), restriction enzymes and T4 DNA ligase were from Boehringer Mannheim GmbH., (Lewes, Sussex, UK). QIAGEN plasmid purification kit was obtained from QIAGEN Ltd. (Dorking, Surrey, UK). Tris-washed phenol (pH >7.6), was from Fisons (Leicestershire, UK). Ethidium bromide, tissue culture components (Dulbecco’s Modified Eagle Medium F-12 Ham (DMEM)/F-12 1:1 mixture, trypsin-EDTA, and Hanks balanced salt solution (HBSS)), poly-L-lysine, luminol, p-coumaric acid and ribonuclease A (RNAase A) were from Sigma Chemical Company (Poole, Dorset, UK). Sterile foetal calf serum (FCS) was obtained from Advanced Protein Products (West Midlands, UK). Disposable filter Sartolab-V150 units were purchased from Sartorius (Goettingen, Germany). Acrylamide and N’ N’-methylenebisacrylamide were from BDH Laboratory Supplies (Leicestershire, UK). Pre-stained molecular weight standards for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and agarose were purchased from Life Technologies Ltd. (Uxbridge, UK). CytoTox 96™ Non-Radioactive Cytotoxicity Assay was from Promega Ltd. (Southampton, UK). [³H] MK801 (28.8 Ci/mmol) was from Dupont (Stevenage, Herts, UK). The expression vector pcDNA 1.1
Amp was obtained from Invitrogen (Netherlands). Ketamine and MK801 maleate were from Research Biochemicals Inc. (Natick, MA; USA). Horseradish peroxidase (HRP)-linked secondary antibody, Hyperfilm™ and [³H] MDL105,519 (85 Ci mmol⁻¹) were purchased from Amersham International (Aylesbury, Bucks., UK). All other drugs were purchased from Tocris Neuramin (Bristol, UK). All other materials were of the highest commercial purity available.

The following peptides were used for antibody and affinity column production by Dr. P.L. Chazot. The peptides NR1 (17-35) amino acid sequence, TRKHEQMFREAVNQANKRHC and NR2D/C amino acid sequence, LGTRRGS AHFSSLESEV were from Immune Systems (Bristol, UK). NR1 (911-920) peptide sequence, LQLCSRHRES, was from Multiple Peptide Systems (San Diego, CA, USA). The multiple antigen peptide (MAP), NR2A/2B (1435-1445)AA, YKKMPSTESDVAAMAP and NR2C (1208-1218) amino acid sequence, RLGAGQGEQAVTVAC were from Peptide and Protein Research (University of Exeter, UK).

GV150,526A and GV196,771A, (both > 99% purity as determined by both mass spectrometry and high performance liquid chromatography (HPLC)) were gifts from GlaxoWellcome (Verona, Italy). NMDA receptor cDNAs were generous gifts from Professors S. Nakanishi (Kyoto, Japan) and M. Mishina (Nigata, Japan). HEK 293 cells were a gift from Professor T. G. Smart (School of Pharmacy, London, UK) and the pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, CA, USA).
2.2 METHODS

2.2.1 PREPARATION OF COMPETENT CELLS

All procedures were performed using a sterile flame technique and the solutions were autoclaved before use. Competent *E. coli* cells were prepared as described in Sambrook *et al.,* (1989). Briefly, *E. coli* cells were grown overnight in media A (Luria-Bertani (LB) media containing, bactotryptone 10 g/l, bactoyeast extract 5 g/l and NaCl 10 g/l supplemented with 10 mM MgSO₄ and 0.2% (v/v) glucose) (10 ml) by incubating at 37°C with vigorous shaking in a Luckham R300 orbital shaker. The overnight culture (0.5 ml) was added to fresh media A (500 ml) and incubated at 37°C, with shaking at 300 rpm for 3-4 h until the optical density (OD) at λ = 600 nm was in the range 0.6 - 0.8. Following a 10 min incubation on ice, the cell culture was centrifuged at 3000 rpm in a Megafuge 1.0 R centrifuge, for 10 min at 4°C and the supernatant was discarded. The pellet was resuspended in media A (0.5 ml) by stirring using a sterile loop. Media B (LB media supplemented with 36% (v/v) glycerol, 12% (w/v) PEG 8000, 12 mM MgSO₄) (2.5 ml) was added to the cell suspension and mixed using a sterile pipette. The competent cells were divided into aliquots of 0.1 ml in precooled eppendorf tubes and stored at -80°C until use.
2.2.2 TRANSFORMATION OF BACTERIAL CELLS

For the transformation of competent *E. coli* cells, a frozen aliquot of the cells (100 μl) were removed from -80°C and thawed on ice for 5 min. The appropriate plasmid DNA (20 ng/μl) was added to the competent cells (100 μl) and mixed gently. The cell mixture was then incubated on ice for 30 min and heat-shocked by placing in a water bath at 42°C for 60 sec. After a 2 min incubation on ice, LB media (section 2.2.1) (900 μl) was added to the transformed cells. Following a 1 h incubation at 37°C the cell suspension (100 μl) was plated onto culture plates prepared with 1.5% (w/v) agar in LB media containing ampicillin (50 μg/ml). The culture plates were incubated at 37°C for 18-20 h in an inverted position.

2.2.3 STORAGE OF COMPETENT *E. COLI* CELLS

Competent *E. coli* cell stocks were prepared by mixing 0.5 ml LB media (section 2.2.1) supplemented with 50% (v/v) sterile glycerol and 50 μg/ml ampicillin with 0.5 ml of the small overnight culture (section 2.2.4.1). The cell culture was immediately added to a cryogenic vial and stored at -80°C until use.
2.2.4 AMPLIFICATION OF PLASMID DNA

2.2.4.1 Preparation of small-scale culture of plasmid DNA

LB media (section 2.2.1) (10 ml) containing ampicillin (50 µg/ml) was added to a 50 ml Falcon tube and inoculated with one isolated colony from the culture plate (section 2.2.2) using a sterile loop. The small culture was incubated for 18-20 h with shaking at 300 rpm at 37°C.

2.2.4.2 Preparation of Large-Scale Culture of Plasmid DNA

LB media (500 ml) containing ampicillin (50 µg/ml) was inoculated with the overnight culture (section 2.2.4.1) (0.5 ml) in a 500 ml flask. The large culture was incubated for 18-20 h at 37°C with shaking at 300 rpm.

2.2.5 PREPARATION OF PLASMID DNA FROM BACTERIAL CELLS

2.2.5.1 Small-scale preparation of plasmid DNA

Small-scale preparation of plasmid DNA was carried out as described in Sambrook et. al., (1989). The small overnight culture (section 2.2.4.1) (1.5 ml) was added to an eppendorf and centrifuged at 12 000 x g (14600 rpm) for 3 min at room temperature using a benchtop
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minicentaur centrifuge. The supernatant was discarded and the pellet was re-centrifuged at 12,000 x g for 30 sec again at room temperature. The remaining supernatant was discarded. Ice-cold 50 mM Tris-HCl, pH 8.0, containing 50 mM glucose and 10 mM EDTA, (TGE)(100 µl) and, 200 µl of alkaline sodium dodecyl sulphate (SDS) solution (10 M NaOH and 10% (w/v) SDS), was added to the pellet and the samples were mixed by inversion of the tubes. Ice-cold 3 M potassium acetate, pH 4.8 (150 µl) was added and the tubes were mixed by vigorous inversion. Following a 5 min incubation on ice, the samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was transferred to a fresh tube. The supernatant was extracted with an equal volume of phenol/chloroform mixture (phenol : chloroform : amyl alcohol, 25:24:1, volume ratio) and centrifuged at 12,000 x g for 2 min at room temperature. The top layer was transferred to a fresh eppendorf and 2 volumes of absolute alcohol was added to precipitate the DNA. After a 15 min incubation on ice, the sample was centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was discarded and the remaining pellet washed with 70% (v/v) ethanol (0.5 ml). The DNA pellet was air-dried for 30 min and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA) (25 µl) and ribonuclease A (RNAase) (40 µg/ml) (1.64 µl). Plasmid DNA was stored at 4°C until use.
2.2.5.2 Harvesting of the large-scale culture and purification of plasmid DNA using QIAGEN™ plasmid maxi-kit

*E. coli* cells were harvested by transferring the large overnight culture (500 ml) (section 2.2.4.2) into two ice-cold JA14 centrifuge tubes and these were centrifuged using a Beckman J2-21 centrifuge at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the remaining pellets were resuspended in ice-cold P1 buffer (50 mM Tris-HCl, pH 8, containing 10 mM EDTA and 100 µg/ml RNAase A) (10 ml). On addition of the P2 solution (200 mM NaOH, 1% (v/v) SDS) (10 ml) the tube was mixed by gentle inversion and incubated at room temperature for 5 min. Chilled P3 buffer (2.55 M potassium acetate, pH 4.8) (10 ml) was added to the centrifuge tube which was mixed by inversion and incubated on ice for 20 min. The solution was centrifuged at 11,500 rpm for 30 min at 4°C and the clear lysate was removed into a fresh JA20 centrifuge tube. A QIAGEN™ 500 tip was equilibrated with QBT buffer (50 mM 3-[N-morpholino]propane-sulphonic acid (MOPS), pH 7.0, containing 750 mM NaCl, 15% (v/v) ethanol and 0.15% (v/v) Triton X-100) (10 ml). The lysate was loaded onto the column and allowed to flow under gravity. The column was washed with QC buffer (50 mM MOPS, pH 7.0, containing 1 M NaCl, 15% (v/v) ethanol) (2 x 30 ml) and QF buffer (50 mM MOPS, pH 8.2, 1.25 M NaCl, 15% (v/v) ethanol) (15 ml), was added to the column to elute the plasmid DNA. Isopropanol (0.7 vol) (10.5 ml) was added to the eluted DNA and the solution was centrifuged at 11,500 rpm for 30 min at room temperature. The DNA pellet was carefully washed with ice-cold 70% (v/v) ethanol (1 ml) and air-dried for
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approximately 30 min. The purified DNA was dissolved in TE buffer pH 8.0 (section 2.2.5.1) (0.5 ml) and stored at 4 °C until use.

2.2.6 SPECTROPHOTOMETRIC QUANTIFICATION OF DNA

The purity and concentration of plasmid DNA was determined by measuring the OD at $\lambda = 260$ nm and at $\lambda = 280$ nm (Sambrook et al., 1989). The ratio of the optical densities at $\lambda = 260$ nm and $\lambda = 280$ nm ($\text{OD}_{\lambda=260\text{ nm}} / \text{OD}_{\lambda=280\text{ nm}}$) should be within the range 1.8 - 2.0. Plasmid DNA concentration was then calculated at $\lambda = 260$ nm. An OD = 1 corresponds to ~ 50 µg/ml for double stranded DNA (dsDNA).

2.2.7 RESTRICTION ENZYME DIGESTION

The appropriate 10 x restriction buffer (2 µl) for the corresponding restriction enzyme was added to DNA (1.0 µg) and kept on ice. The restriction enzyme (1 unit, 1 µl) was added to this solution and sterile water was used to make the final volume to 20 µl. The solution was then mixed and centrifuged at 12 000 x g (14600 rpm) for 10 sec. The digestion reaction was incubated at 37°C for 1 h and terminated by placing on ice. DNA samples (5 µl) were analysed by flat-bed agarose gel electrophoresis (section 2.2.8). For larger amounts of DNA, the reaction was scaled appropriately.
2.2.8 FLAT-BED AGAROSE GEL ELECTROPHORESIS

Flat-bed agarose gel electrophoresis was performed using Flowgen minigel apparatus MF1070, which were assembled with a well-forming comb and aluminium plates (Sambrook et al., 1989). A stock solution of 10 x Tris-borate buffer containing, 0.9 M Tris base, 0.9 M boric acid and 40 mM EDTA) (TBE) was diluted 1:10 with sterile water and agarose (0.9% (w/v)) was added. The agarose solution was autoclaved, for 40 min and stored at 60-70°C until use.

Ethidium bromide (10 mg/ml) (5 µl) was added to the precooled agarose solution (40 ml) which was immediately poured into the mini-gel apparatus and allowed to polymerise at room temperature for 20-30 min. Running buffer (1 x TBE) (50 ml) was poured over the gel and the comb and aluminium plates were then carefully removed. DNA samples for analysis were prepared by the addition of 6 x gel-loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) glycerol and 60 mM EDTA, pH 8.0) (2 µl) to the DNA (100 ng) and sterile water added to final volume of 12 µl. Pre-stained standards containing, DNA marker molecular weight II (0.12-23.1 kbp, λ DNA, Hind III digested) (10 µl), 6 x gel-loading buffer (26 µl) and sterile water (124 µl) (12 µl), and DNA samples were loaded into the wells. Electrophoresis was performed at a constant voltage of 50 V for 45-60 min at room temperature. The gel was visualised using a Flowgen ultraviolet (UV) transilluminator, (TF-10M) at λ = 312 nm. Agarose gels were photographed under UV illumination using a Polaroid DS34 instant camera with Polaroid 665 positive/negative instant film.
2.2.9 DNA LIGATIONS

2.2.9.1 Preparation of dialysis tubing

Dialysis tubing (visking size 11/4") which was approximately 5 cm in length was boiled in sterile water for 5 min and allowed to cool. The tubing was stored in water before use.

2.2.9.2 Isolation and electroelution of DNA fragments

The foreign DNA (8 µg) to be ligated into the plasmid vector was digested with the appropriate restriction enzymes in a final volume of 40 µl (section 2.2.7). The digested DNA was analysed by 0.8% (w/v) flat-bed agarose gel electrophoresis as described in section 2.2.8, but with the exception that the gel was cast using a 50 µl well comb and 40 µl of DNA samples were loaded into each well. Gel electrophoresis was performed at room temperature for 2 h at 50 V. The desired DNA fragment was excised from the agarose gel by using a sterile scalpel blade under UV illumination and the remaining gel was discarded (Sambrook et al., 1989). The gel slice containing the DNA band of interest was placed into the hydrated dialysis bag (section 2.2.9.1), which contained TE buffer pH 8.0 (section 2.2.5.1). Both ends of the tubing were secured with plastic clips. The dialysis bag containing the piece of agarose was placed horizontally in the base plate of the mini-gel apparatus and running buffer (1 x TBE) (50 ml) was added. Electroelution of the DNA fragment was performed at 50 V constant voltage for 3-4 h at room temperature. The
migration of the DNA from the agarose gel piece into the TE buffer pH 8.0, was confirmed by visualising under UV illumination. The TE buffer pH 8.0, containing the electroeluted DNA fragment was removed into a fresh eppendorf.

2.2.9.3 Ethanol precipitation of DNA

DNA precipitation was performed by the addition of 3 M sodium acetate pH 5.8, (0.1 vol) and absolute ethanol (2 vol) to the DNA solution (section 2.2.9.2), which was incubated at -20 °C for at least 2 h (Sambrook et al., 1989). The DNA sample was centrifuged at 12 000 x g (14600 rpm) for 15 min at 4 °C and the supernatant was discarded. Ice-cold ethanol 70% (v/v) (200 μl) was added to the DNA pellet and it was centrifuged at 12 000 x g for 15 min at 4 °C. The supernatant was removed and the DNA pellet was air-dried for approximately 30 min and resuspended in TE buffer, pH 8.0 (section 2.2.5.1) (20 μl). Any remaining agarose from the electroelution of the DNA (section 2.2.9.2) was removed by heating the sample to 37°C for 5 min. The sample was centrifuged at 12 000 x g for 5 min at room temperature and the supernatant was removed carefully into a fresh eppendorf. The DNA sample was analysed by restriction enzyme digestion and the approximate concentration was determined by agarose flat-bed gel electrophoresis, in combination with DNA pre-stained markers as in sections 2.2.7 and 2.2.8.
2.2.9.4 Phenol/chloroform extraction of DNA fragments

Vector DNA to be used in ligation reactions was purified using phenol/chloroform extraction (Sambrook et al., 1989) after restriction enzyme digestion. An equal volume of Tris-washed phenol was added to the DNA solution and the sample was vortexed for 1 min. After a 5 min centrifugation at 12 000 x g (14600 rpm) at room temperature, the top layer was removed into a fresh eppendorf and chloroform (1 vol) was added. The sample was again vortexed for 1 min and centrifuged at 12 000 x g for 5 min at room temperature. Finally, the upper layer was retained and re-extracted with chloroform (1 vol). The DNA was precipitated with absolute ethanol as described in section 2.2.9.3.

2.2.9.5 DNA ligation reactions

The DNAs to be ligated were digested with appropriate restriction enzymes to produce complementary sticky ends as described in section 2.2.7. The foreign DNA was electroeluted from the agarose gel (section 2.2.9.2) and the vector DNA was purified using phenol/chloroform extraction (section 2.2.9.4). Both foreign and vector DNA were concentrated using ethanol precipitation (section 2.2.9.3) and the DNA concentration was estimated using agarose flat-bed gel electrophoresis, in combination with DNA pre-stained standards (section 2.2.8). For the ligation reaction, vector DNA (100-150 ng) was mixed with a range of concentrations of foreign DNA (i.e. vector DNA : foreign DNA, 1:1, 1:3 and 1:10 volume ratio) in a sterile thin-walled PCR tube (Sambrook et al., 1989). Ligase
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10 x buffer containing final concentration of 20 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 10 mM dithiothreitol (DTT) 10 mM adenosine triphosphate (ATP) (2 µl), and T4 DNA ligase (1 unit, 1 µl), was added to the DNA mixture. Finally, water was added to a final volume of 10 µl. The reaction was mixed gently and incubated at 16°C for 16-20 h. The ligation mixture (5 µl) was transformed into competent *E. coli* cells as described in section 2.2.2. Small-scale cultures and preparation of plasmid DNA were performed as in sections 2.2.4.1 and 2.2.5.1.

2.2.10 PREPARATION OF MAMMALIAN CELL CULTURE MEDIA

2.2.10.1 Preparation of DMEM/F12 media + L-Glutamine

All procedures were performed using sterile conditions. Powdered Dulbecco's Modified Eagle Medium/F-12 Ham (DMEM/F-12 1:1 mixture)(15 g/l) containing, L-glutamine (0.365 g/l) and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES) was mixed with sterile water (800 ml). The mixture was supplemented with 100 ml of 10% (v/v) foetal calf serum (FCS), 40 ml of 7.5% (w/v) NaHCO₃ (final 3.0 g/l) and penicillin (500 IU/ml) / streptomycin (500 µg/ml) solution (20 ml). The final volume was made up to 1 l with sterile water and the pH of the media was adjusted to pH 7.6 using NaOH (10 M). The media was filter-sterilised using a 0.2 µm Sartorius Sartolab-V150 filter unit and stored at 4°C until use.
2.2.10.2 Preparation of DMEM/F12 media - L-Glutamine

All procedures were carried out using sterile conditions. Liquid DMEM/F12 1:1 mixture containing, 15 mM HEPES (700 ml) but lacking L-glutamine was mixed with 100 ml of 10% (v/v) FCS, 24 ml of 7.5% (w/v) NaHCO₃ (final 3.0 g/l) and penicillin (500 IU/ml) / streptomycin (500 μg/ml) solution (20 ml). The final volume was made up to 1 l using sterile water and the pH was adjusted to pH 7.6 using NaOH (10 M). The media was filter-sterilized as in section 2.2.10.1 and stored at 4°C until use.

2.2.11 SUBCULTURING OF HEK 293 CELLS

HEK 293 cells were grown in 250 ml Greiner tissue culture flasks at 37°C in 5 % CO₂ in DMEM/F12 media containing, L-glutamine (section 2.2.10.1) in an IR 1500 automatic CO₂ incubator. Every two to three days cells were subcultured by the removal of the old media and then washed with prewarmed Hanks buffered salt solution (HBSS), containing sodium carbonate (~ 5 ml). Following a 1 min incubation in trypsin-EDTA (trypsin 0.5 g/l and EDTA 0.2 g/l) (2 ml) at 37°C, DMEM/F12 media containing L-glutamine (10 ml) was added to the cells. The cells were then separated by gentle pipetting. Finally, the cell suspension (2 ml) was added to a fresh flask and a further 10 ml of DMEM/F12 media containing L-glutamate was added to the new flask, which was incubated at 37°C in 5% CO₂.
2.2.12 PREPARATION OF GLYCEROL AND NEW STOCKS OF HEK 293 CELLS

HEK 293 cell stocks were prepared by subjecting the cells to a trypsin-EDTA (section 2.2.10) (4 ml) dissociation for 1 min at 37°C and 20 ml of DMEM/F12 media containing L-glutamine was added. The cells were pelleted by centrifugation at 200 x g (1300 rpm) for 5 min at 4°C. The pellet was resuspended in DMEM/F12 media containing L-glutamine (4.8 ml) supplemented with, 0.6 ml of 10% (v/v) FCS and dimethyl sulphoxide (DMSO) (0.6 ml). The cell suspension was immediately divided into three cryogenic vials and stored at -80°C for 24 h and then transferred to liquid nitrogen.

For the preparation of a new culture, a single cryogenic vial of frozen HEK 293 cells was thawed at 37°C. Cells were pelleted by centrifugation at 200 x g for 5 min at 4 °C and resuspended in DMEM/F12 media containing L-glutamine (15 ml). The cells were added to a tissue culture flask which was incubated at 37°C in 5% CO₂ and cultured as described in section 2.2.11.

2.2.13 CALCIUM PHOSPHATE PRECIPITATION-MEDIATED TRANSFECTION OF HEK 293 CELLS

HEK 293 cells were transfected by the calcium phosphate precipitation method (Gorman et al., 1990). Cells were subcultured 24 h prior to the transfection to a density of ~ 4 x 10⁶ cells per flask as in section 2.2.11. On the day of the procedure the media was removed 3h prior to the transfection and replaced with 10 ml of DMEM/F12 media lacking L-
glutamine. The cells were incubated at 37 °C in 7.5% CO₂.

For the transfection of the cells, two tubes were prepared. These were tube a containing 440 μl 1/10 TE buffer (section 2.2.5.1) (TE buffer, pH 8.0 diluted 1:10 in sterile water), 10 μl DNA (1 μg/μl) (i.e. pCISNR1 (total volume of DNA 10 μl), pCISNR1: pCISNR2 in a 1:3 volume ratio, total volume of DNA 10 μl) and tube b containing, 2 x 50 mM HEPES buffered saline (HBS), pH 7.12 with 280 mM NaCl and 1 mM Na₂HPO₄ (500 μl). Calcium chloride (2.5 M) (50 μl) was heated to 37° C and was slowly added to tube a and mixed well. The contents of tube a were added to tube b at a rate of 1 drop per 5 sec and the mixture was pipetted up and down until the solution appeared cloudy. The contents of tube b were added slowly to the media over the HEK 293 cells and the media was swirled to distribute the precipitate evenly. Following a 3 h incubation at 37°C at 7.5% CO₂, the media from the cells was aspirated. Glycerol (15% (v/v)) in phosphate buffered saline (PBS) containing, 4 mM Na₂HPO₄, 1.7 mM KH₂PO₄, pH 7.4, 137 mM NaCl and 107 mM KCl (1.5 ml) was gently spread over the cells and incubated for 30 sec at room temperature. The glycerol solution was removed and the cells rinsed with DMEM/F12 media lacking L-glutamine (~5 ml). Fresh L-glutamine-free DMEM/ F12 media (10 ml) was added. Cells transfected with pCISNR1-1a/pCISNR2A and pCISNR1-1a/pCISNR2B were grown in the presence, of 1 mM ketamine post-transfection to prevent NMDA receptor-mediated cytotoxicity. Finally the cells were incubated at 37°C for 24 h at CO₂ level of 5% and were harvested as described in section 2.2.14.1.
2.2.13.1 Preparation of intact HEK 293 cells

When transfected HEK 293 cells (section 2.2.13) were to be processed as intact cells, cells were subcultured 1 h following glycerol shock as described in section 2.2.11, but with the exception that 20 ml of DMEM/F12 media lacking L-glutamine was added to the flask. The cells (2.2 ml) were plated onto poly-L-lysine treated (50 μg/ml) 35-mm Nunc petri dishes. Cells transfected with pCISNR1-1a/pCISNR2A and pCISNR1-1a/pCISNR2B were grown in the presence of 1 mM ketamine.

2.2.14 MEMBRANE PREPARATION

2.2.14.1 Harvesting and membrane preparation of HEK 293 cells

Transfected HEK 293 cells (section 2.2.13) were harvested 24 h post-transfection. Cells were scraped into the culture media using Greiner cell scrapers and placed into ice-cold JA20 centrifuge tubes. The flask was washed with ice-cold 50 mM Tris-citrate pH 7.1, containing 5 mM EDTA and 5 mM EGTA, and the washings were added to the centrifuge tubes. The cell suspensions were centrifuged for 5 min at 5000 rpm at 4°C. The pellet collected was homogenised in ice-cold Tris-citrate pH 7.1, using a Dounce glass/glass homogeniser (15 strokes). The homogenate was re-centrifuged at 11500 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was homogenised and centrifuged as before. The final cell pellet was then resuspended in ice-cold Tris-citrate pH 7.1 (2 ml)
(~50-100 µg protein) and was either assayed immediately for radioligand binding activity (section 2.2.20) or alternatively was analysed for the expression of the NMDA receptor subunits by immunoblotting using the appropriate anti-NMDA receptor subunit-specific antibodies (section 2.2.18.2).

2.2.14.2 Adult rat P2 membrane preparation

Male Wistar (250-300 g) rats were stunned and decapitated. The forebrain tissue was dissected and rapidly snap-frozen in liquid nitrogen. A Potter glass-teflon homogenizer was used (6000 rpm, 15 strokes) to homogenise the tissue at 4 °C in all instances. The tissue was thawed (20 g) and homogenised in ice-cold 50 mM Tris-citrate pH 7.1, (section 2.2.14.1) containing, 320 mM sucrose, bacitracin (5 µg/ml), benzamide HCl (5 µg/ml), phenylmethyl sulphonyl fluoride (PMSF) (33 µg/ml), ovomucoid trypsin inhibitor (5 µg/ml) and soybean trypsin inhibitor (5 µg/ml) (9 vol (w/v)). The homogenate was centrifuged at 2200 rpm for 10 min at 4 °C and the supernatant was stored on ice. The pellet was rehomogenised in ice-cold Tris-citrate pH 7.1 containing the protease inhibitors (4.5 vol (w/v)) and recentrifuged at 4 °C for 10 min at 2200 rpm. The supernatant from the first and second centrifugation were pooled together and were centrifuged at 15000 rpm at 30 min at 4 °C. The supernatant was discarded and the pellet was homogenised in Tris-citrate buffer pH 7.1 (4.5 vol (w/v)). The homogenate was centrifuged at 14000 rpm at 4 °C for 30 min and the pellet collected was homogenised in Tris-citrate pH 7.1 (4.5 vol (w/v)). The homogenate was snap-frozen in liquid nitrogen, thawed, re-homogenised in Tris-citrate pH
7.1 (4.5 vol (w/v)) and again centrifuged at 14000 rpm for 30 min at 4°C. This freeze-thaw cycle was repeated 5 times. The final pellet was resuspended in Tris-citrate pH 7.1, to a final protein concentration of 1 mg/ml and aliquots of 0.1 ml were divided into precooled eppendorf tubes. The membranes were either assayed for radioligand binding activity (section 2.2.20), prepared for immunoblotting (section 2.2.18.2) or stored at -20°C until use.

Frozen rat P2 membranes prior to use were thawed and homogenised in ice-cold Tris-citrate pH 7.1 using a Dounce glass/glass homogeniser (15 strokes). The homogenate was centrifuged at 11500 rpm for 30 min at 4°C. The pellet was resuspended in ice-cold Tris-citrate pH 7.1 (2 vol (w/v)) (~100 μg protein) and assayed immediately for radioligand binding activity (section 2.2.20) or prepared for immunoblotting (section 2.2.18.2).

2.2.15 DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration was determined using the method of Lowry et al., (1951) employing bovine serum albumin (BSA) as the standard protein. A stock solution of BSA (4 mg/ml) was serially diluted in water, to give a range of standard BSA concentrations from 0 to 100 μg/ml. Reagent A (2% (w/v) sodium carbonate, 0.1 M NaOH and 5% (w/v) SDS), reagent B (2% (w/v) sodium potassium tartrate) and reagent C (1% (w/v) copper sulphate) were mixed in a volume ratio of A (50) : B (1) : C (1). To both the BSA standards and the unknown protein samples (50 μl) 0.5 ml of the mixture of reagent A, B and C was added, each sample was vortexed and incubated at room temperature for 10 min. All
samples were assayed in triplicate. On the addition of 50 µl of Folin-Ciocalteau phenol reagent (1 M, 1:1 mix of Folin reagent and water) each sample was mixed and 500 µl of water was added. The samples were incubated at room temperature for 30 min. The OD at \( \lambda = 750 \text{ nm} \) was determined for each sample using a UV/VIS spectrophotometer. A calibration curve was plotted of OD at \( \lambda = 750 \text{ nm} \) for the BSA samples. This was then used to determine the unknown protein concentration.

2.2.16 DETERMINATION OF CYTOTOXICITY AND THE TRANSFECTION EFFICIENCY OF TRANSFECTED HEK 293 CELLS, USING A CYTOTOX 96™ NON-RADIOACTIVE CYTOTOXICITY ASSAY

The CytoTox 96™ assay is a colorimetric assay which quantitatively measures lactate dehydrogenase (LDH) released upon cell lysis. A sample of media (1 ml) was collected 20 h post-transfection from transfected HEK 293 cells (section 2.2.13) and from control cells which were untransfected HEK 293 cells. These were termed, experimental samples. The remainder of the cells in the untransfected and transfected flasks were incubated at -80°C for 30 min and then at 37°C until the media had thawed. A 1 ml fraction of media was collected from these flasks which were the total samples since the freeze-thaw cycle resulted in complete cell lysis. All samples (1 ml) were centrifuged at room temperature for 1 min at 13 000 x g (15200 rpm) and the supernatant was transferred into fresh eppendorfs. Samples were diluted with HBSS (section 2.2.11), i.e. total sample : HBSS 1:10 and experimental sample : HBSS 1:3. Experimental, total and blank (containing HBSS only)
samples (50 μl) were added in triplicate to a 96 well enzyme-linked-immunosorbent assay (ELISA) plate. Reconstituted substrate mix (tetrazolium salt and diaphorase) (50 μl) was added to all samples and the assay plate was covered with aluminium foil. Following a 30 min incubation at room temperature, stop solution (1 M acetic acid) (50 μl) was added. The OD of each sample at λ = 490 nm was recorded using a Dynatech minireader. The OD at λ = 490 nm for the control samples was subtracted from all absorbance values of experimental and total samples from transfected HEK 293 cells. The percentage cytotoxicity was calculated from the following equation using the corrected values.

\[
\frac{\text{Mean absorbance from experimental flasks}}{\text{Mean absorbance from total flasks}} \times 100 = \% \text{ Cytotoxicity}
\]

The percentage cytotoxicity was also used as a quantitative measure of the transfection efficiency when cytotoxic plasmid DNAs, i.e. pCISNR1-1a/pCISNR2A and pCISNR1-1a/pCISNR2B were transfected into HEK 293 cells (Cik et al., 1993).

2.2.17 PEPTIDE AFFINITY PURIFICATION OF ANTIBODIES

Specific peptide sequences of NMDA receptor subunits were conjugated to carrier proteins which were then used to generate polyclonal antibodies in rabbits (Chazot et al., 1994; 1997a,b). Antibodies were purified using affinity columns using the respective peptide that
was used to generate the anti-peptide antibodies linked to a Sepharose column (Stephenson and Duggan, 1991). Both the immunisation of rabbits and preparation of peptide affinity columns was performed by Dr. P.L. Chazot.

For the purification of anti-peptide polyclonal antibodies a Sepharose column (1 ml) linked to the appropriate peptide was equilibrated with phosphate buffered saline (PBS) containing 10.1 mM Na₂HPO₄·12H₂O, pH 7.4, K₂HPO₄, 136.9 mM NaCl and 2.7 mM KCl at a rate of 40 ml/h for 1 h using a 2232 Microperpex S peristaltic pump. Immune serum (5 ml) was applied to the column using a pasteur pipette and recirculated at a rate of 40 ml/h for 2 h at room temperature or overnight at 4°C. Unbound immune serum was discarded and the column was washed with PBS for 2 h at a rate of 40 ml/h. The bound antibody was eluted from the column with 50 mM glycine/HCl pH 2.3 (10 ml) at a rate of 10 ml/h. The purified antibody fractions (1 ml) were collected in eppendorfs containing 1 M Tris (20 µl) to give a final pH 7.4, in a Biorad 2110 fraction collector at a rate of 10 ml/h. The OD at \( \lambda = 280 \) nm of the antibody fractions was determined and the concentration of the antibodies was calculated using the Beer Lambert law,

\[
\frac{A}{\varepsilon L} = C
\]

where;

- \( C \) is the concentration of antibody,
- \( A \) is the absorbance of the antibody at \( \lambda = 280 \) nm,
- \( \varepsilon \) is the molar extinction coefficient and
- \( L \), is the path length.
The yields of the purified antibodies were in the range 0.2-0.6 mg protein/ml. The antibody-containing fractions were pooled and dialysed as in section 2.2.17.1. The affinity columns were regenerated with PBS at a rate of 40 ml/h for 30 min at room temperature. The column was stored with the addition of 0.025% (w/v) NaN₃ at 4°C until further use.

2.2.17.1 Dialysis of purified antibodies

A dialysis tubing was washed throughly with water and was clipped at one end with a dialysis clip. The purified antibody fraction from section 2.2.17 was placed inside the tubing which was secured at the other end with a clip. Dialysis was performed overnight at 4°C in PBS (section 2.2.17) containing, 0.025% (w/v) NaN₃. The concentration of the antibody was recalculated by measuring the OD at λ = 280 nm as in section 2.2.17.

2.2.18 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Immunoblotting was carried out essentially as described by Duggan et al., (1991), using SDS/PAGE in 7% polyacrylamide mini-slab gels under reducing conditions with a final antibody concentration of 1 µg/ml.
2.2.18.1 Preparation of resolving gel

The resolving gel (7%) was prepared by mixing water (31 ml) with 1 x electrophoresis buffer pH 8.8 which contained, 50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS (15 ml), N,N,N′,N′-tetramethylethylenediamine (TEMED) (30 μl), 30% (w/v) mix of acrylamide and N,N′-methylenebisacrylamide (14 ml). The solution was degassed for 15 min and 10% (w/v) ammonium persulphate (APS) (300 μl) was added. The polyacrylamide solution was immediately poured into a Biotech gel caster holding 10 gels, using gel plates of 10 x 8 cm and spacers of 1 mM width. Saturated water/butanol solution (100 μl) was added over the top of each gel. The gels were covered with foil and were allowed to polymerise for 60 min at room temperature. Gels were individually wrapped in tissue and stored at 4°C until use.

2.2.18.2 Chloroform/methanol method for protein precipitation and preparation of protein samples for SDS-PAGE

Protein samples for SDS-PAGE (from sections 2.2.14.1 and 2.2.14.2) were precipitated using chloroform/methanol precipitation described as follows. To the protein samples (25-50 μg determined as in section 2.2.15), methanol (4 vol) was added and the samples were vortexed and centrifuged briefly at room temperature at 12 000 x g (14600 rpm). Chloroform (1 vol) was added to the samples which were vortexed and centrifuged at 12 000 x g at room temperature for 30 sec. To each of the samples water (3 vol) was added,
which were again vortexed and centrifuged at room temperature at 12,000 x g for 60 sec. The upper layer was carefully discarded and methanol (1 vol) was added to each of the samples. The samples were centrifuged at 12,000 x g at room temperature for 4 min. The supernatant was removed and the samples were vacuum-dried. The dried protein pellet was resuspended by vortexing in sample buffer containing 30 mM NaH$_2$PO$_4$, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and, 7.5% (w/v) SDS (5 μl), 100 mM DTT (1.5 μl) and water to a final volume of 15 μl. The samples were boiled in a water bath for 3 min and then centrifuged at 12,000 x g for 30 sec at room temperature before analysis by SDS-PAGE section 2.2.18.3.

### 2.2.18.3 SDS-polyacrylamide gel electrophoresis

The resolving mini-slab gel (section 2.2.18.1) was clamped into a Hoefer Mighty Small II vertical slab SE250 unit. The stacking gel was prepared by mixing water (2.3 ml) with stacking gel buffer containing, 0.5 M Tris-glycine, pH 6.8, 8 mM EDTA and 0.4% (w/v) SDS (1 ml), 30% (w/v) mix of acrylamide and N,N'-methylenebisacrylamide (650 μl) and TEMED (5 μl) which was degassed for 10-15 min. APS (100 mg/ml) (80 μl) was added to the degassed stacking gel solution and this was immediately poured into the mini-slab gel above the resolving gel. A welled comb was inserted into the stacking gel. After the polymerisation of the gel, the comb was carefully removed and the wells were washed with water. Electrode buffer pH 8.8, containing 50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS (~ 200 ml) was poured into the wells and into the base of the
electrophoresis unit. Protein samples (15 µl) (section 2.2.18.2) and pre-stained standards (protein molecular weight range of 200-6.5 kDa, in storage buffer containing, 50% glycerol, 300 mM NaCl, 100 mM DTT, 10 mM Tris, 2 mM EDTA and 3 mM NaN₃) (15 µl) were loaded into the wells of the stacking gel using a Hamilton syringe. Electrophoresis was carried out at a constant current of 15 mA for ~ 2 h until the appropriate pre-stained molecular weight marker (25 kDa) was at the bottom of the gel. This allowed a better resolution for NMDA receptor subunits. SDS-PAGE gels were immunoblotted as described in section 2.2.19.

2.2.19 IMMUNOBLOTTING OF SDS-PAGE GELS

After SDS-PAGE (section 2.2.18.3), the proteins from the gel were transferred to nitrocellulose membranes. A transfer cassette sandwich was constructed with the following order of components each of which had been pre-equilibrated in transfer buffer (which contained, 25 mM Tris pH 8.4, 192 mM glycine and 20% (v/v) methanol) sponge, two sheets of blotting paper and nitrocellulose membrane. The SDS-PAGE gel, two sheets of blotting paper and a final piece of sponge was added to the transfer cassette sandwich. On the addition of each component to the transfer cassette air bubbles were carefully removed by pressing each layer with a pasteur pipette. Proteins were transferred at a constant voltage of 50 V for 2.5 h using a Hoefer TE series Transphor transfer tank containing transfer buffer at room temperature.

Following the transfer of the proteins, the nitrocellulose membrane was briefly rinsed with
Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.4) and incubated with blocking buffer which was TBS pH 7.4 containing 5% (w/v) dried milk and 0.02% (v/v) Tween-20 (15 ml) for 1 h at room temperature, or overnight at 4°C with gentle shaking using a Luckham R100 Rotatest Shaker. After blocking of the non-specific antibody sites the nitrocellulose membranes were washed with ~10 ml of TBS, pH 7.4. The appropriate affinity-purified primary antibodies (section 2.2.17.1) were diluted in incubation buffer, which was TBS, pH 7.4 containing 2.5% (w/v) dried milk to a final concentration 1 μg/ml. The nitrocellulose membranes were incubated with the diluted primary antibody solution (10 ml) for 1 h at room temperature with gentle shaking.

After the incubation in primary antibody nitrocellulose membranes, were washed four times in wash buffer containing, 50 mM TBS pH 7.4, 2.5% (w/v) dried milk and 0.2% (v/v) Tween-20 (10 ml) at 10 min intervals with gentle shaking at room temperature.

Nitrocellulose membranes were then incubated with horseradish peroxidase (HRP) labelled anti-rabbit secondary antibody, at a dilution of 1/2000 in incubation buffer (10 ml). The membrane was incubated for 1 h at room temperature with gentle shaking. The unbound secondary antibody was removed by washing the membrane as described above.

The nitrocellulose membrane was drained of excess wash buffer and immunoreactive bands on the nitrocellulose membranes were developed by processing in a solution containing, 68 mM p-coumaric acid (100 μl), 1.25 mM luminol (10 ml) and 30% H₂O₂ (6 μl) for 1 min at room temperature. After removal of the reagents the immunoblot was wrapped in cling film and placed in a film cassette. The immunoblot was exposed to Hyperfilm™ for various times (1-10 min). The film was then developed in Kodak D-19 Developer until the
immunoreactive bands were visible and fixed in Kodak Unifix for 5 min at room temperature.

2.2.20 RADIOLIGAND BINDING ASSAYS

[\(^{3}\text{H}\)] MDL105,519 radioligand binding assays were performed using a rapid filtration assay with 1 mM glycine for the determination of non-specific binding and an incubation time of 90 min at 4 °C (Chazot et al., 1998). [\(^{3}\text{H}\)] MK801 binding assays were performed in the presence of 10 µM glutamate, using a polyethylenimine filtration assay, with 10 µM TCP to define non-specific binding and an incubation time of 3 h at room temperature, as described in Chazot and Stephenson, (1997b).

2.2.20.1 [\(^{3}\text{H}\)] MDL105,519 competition assays

Well-washed HEK 293 cells homogenates (section 2.2.14.1) (50-100 µg protein) (100 µl) or adult rat P2 membranes (section 2.2.14.2) (100 µg protein) (100 µl) in 50 mM Tris-citrate buffer pH 7.1, containing 5 mM EDTA and 5 mM EGTA were incubated with 1 nM [\(^{3}\text{H}\)] MDL105,519 (20 µl), and with either 20 µl of buffer or displacing drug in the concentration range 1.0 x 10\(^{-11}\) - 1.0 x 10\(^{-3}\) M, to a final volume of 200 µl for 90min at 4°C. All concentration points were performed in triplicate. Non-specific binding was defined using 1 mM glycine dissolved in Tris-citrate pH 7.1. Bound ligand was collected by rapid filtration using a Brandel cell harvester onto glass fiber filters (GF/B) which had been
presoaked in 50 mM potassium dihydrogen phosphate, pH 7.4, for at least 30 min at 4°C. The filters were washed rapidly three times with ice-cold 50 mM potassium dihydrogen phosphate, pH 7.4, (3 x 3 ml). The filters were placed in minivials and Optiphase 'safe' scintillation fluid (4 ml) was added to each vial. These were incubated for 16-24 h at room temperature and radioactivity was quantified using a Beckman LS 500 CE scintillation spectrophotometer with a counting time of 3 min per vial.

Stock solutions of GV150,526A and GV196,771A were dissolved in 10 mM KOH and 1% (v/v) DMSO respectively and subsequent dilutions for both in 50 mM Tris-citrate, pH 7.1. All other displacing drugs studied (MDL105,519, L689,560, 7-chloro-4-hydroxy-3-(3-phenoxy)-phenyl-2(/H)quinolone (L701,324), 5,7 DCKA and 7-CLKA), were dissolved in DMSO at a final stock concentration of 0.1% (v/v) and were subsequently diluted in 50 mM Tris-citrate, pH 7.1. [³H] MDL105,519 radioligand displacement binding assays using both GV150,526A and GV196,771A were carried out using borosilicate glass tubes, whereas competition binding experiments using all other displacing drugs were performed in polypropylene tubes.

2.2.20.2 [³H] MDL105,519 saturation assays

For [³H] MDL105,519 saturation experiments, well-washed HEK 293 cells homogenates (sections 2.2.14.1) (50-100 μg protein) (100 μl) were incubated in Tris-citrate, pH 7.1, (section 2.2.20.1) in the presence of a range of concentrations of [³H] MDL105,519 i.e. 0.2-15 nM (20 μl) to a final volume of 200 μl. All concentration points were performed in
triplicate. The incubation was performed in polypropylene tubes for 90 min at 4°C. Non-specific binding was defined using 1 mM glycine. The assay was terminated by rapid filtration as described in section 2.2.20.1.

2.2.20.3 \[^{3}\text{H}\] MK801 radioligand binding assays

Well-washed HEK 293 cells homogenates (section 2.2.14.1) (50-100 μg protein) (100 μl), were incubated with 20 μl of L-glutamate (10 μM), 20 μl of \[^{3}\text{H}\] MK801 (40 nM) in Tris-citrate, pH 7.1, to a final volume of 200 μl. The incubation was performed in polypropylene tubes for 3 h at room temperature. Bound ligand was collected by rapid filtration using a Brandel cell harvester as in section 2.2.20.1, with the exception that glass fiber filters (GF/B) were presoaked in 1% (v/v) polyethyleneimine for at least 30 min at room temperature. All concentration points were performed in triplicate. Non-specific binding was defined using 10 μM TCP dissolved in Tris-citrate, pH 7.1.

2.2.20.4 \[^{3}\text{H}\] MDL105,519 radioligand binding assays using intact HEK 293 cells

HEK 293 cells processed as intact cells as described in section 2.2.13.1 (0.1 mg) were washed six times with 2 ml of ice-cold Lockes buffer which contained, 5 mM HEPES, pH 7.4, 154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 1.0 mM MgCl₂ and 5.6 mM glucose. Washed intact cells were incubated in Lockes buffer pH 7.4, with 1 nM \[^{3}\text{H}\] MDL105,519 (200 μl) to a final volume of 1 ml for 90 min at 4°C with gentle shaking. The incubation was terminated by three rapid 2 ml washes with ice-cold Lockes buffer. After
the dishes were allowed to dry, the cells were solubilised overnight with 0.2 M NaOH (1 ml) followed by neutralisation with 2 M HCl (100 µl). An aliquot of the intact cells (100 µl) was retained for the determination of the concentration of protein (section 2.2.15) and the remainder (800 µl) used for quantification of radioactivity as in section 2.2.20.1. Non-specific radioligand binding was defined using 1 mM glycine dissolved in Locke's buffer pH 7.4.

2.2.21 ANALYSIS OF RADIOLIGAND BINDING ASSAY DATA

2.2.21.1 Data analysis for competition studies

Results from the displacement studies were analysed by non-linear least squares regression using Graph PAD prism for both a one-site and a two-site binding model. The displacement results were analysed by a sigmoidal dose response curve with a fixed or variable slope. The F-test was used to assess whether the one-site or the two-site competition model best fit the data (p<0.05). The IC$_{50}$ values for competition curves fitted to a one-site competition model, were calculated from the following equation,

\[
\frac{A + (B - A)}{1 + 10^{(X - Log(C_{50}) nH)}} = Y
\]
where;

A and B = the minimum and maximum percentage specific binding respectively

Y = specific binding at a fixed concentration of displacing drug

X = \log_{10} \text{concentration of the displacer}

IC_{50} = \text{concentration of the displacer which inhibits 50\% of the specific binding of the radioligand}

The IC_{50} values for competition curves fitted to a two-site competition model were calculated from,

\[
A + (B - A) \frac{\text{Fraction 1} \left[ \frac{1 - \text{Fraction}}{1 + 10^{X - \log IC_{501}}} + \frac{1 - \text{Fraction}}{1 + 10^{X - \log IC_{502}}} \right]}{\text{Fraction 1} + \text{Fraction 2}} = Y
\]

where; A, B, X and Y are as above, (1) and (2) = the high and low affinity sites.

For the one-site and two-site binding models, the apparent inhibition constants \( (K_i) \) were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973),

\[
\frac{IC_{50}}{L} = K_i \left(1 + \frac{L}{K_D}\right)
\]
where;

\[ [L] = [\text{^3}H] \text{MDL105,519 concentration} \]

\[ K_D = \text{Dissociation constant determined from saturation binding of [^3H] MDL105,519 (section 2.2.20.2) to the appropriate NR1 or NR1-1a/NR2 combinations (Chazot et al., 1998).} \]

2.2.21.2 Data analysis for saturation studies

Results from the saturation studies were analysed by non-linear least squares regression analysis using Graph PAD prism. The saturation data were analysed by either the one-site or two-site binding hyperbola. The F-test was used to assess whether the one-site or the two-site model best fit the data (p<0.05). All saturation curves of \[^3H\] MDL105,519 bound to NR1 or NR1-1a/NR2 subunits were best fit to a one-site hyperbola. The \( K_D \) values for saturation curves fitted to a one-site hyperbola were calculated from the following equation,

\[
\frac{B_{\text{max}}X}{K_D + X} = Y
\]

where;

\( Y = \text{specific [}^3\text{H] MDL105,519 bound} \)

\( X = \text{concentration of [}^3\text{H] MDL105,519} \)

\( B_{\text{max}} = \text{maximum number of binding sites} \)
Saturation data was fit to the line by linear regression using Graph PAD prism for the
generation of Rosenthal transformations.

\[ f(x) = ax + b \]

where;

\( f(x) \) = specific \[^3\text{H}\] MDL105,519 bound /\[^3\text{H}\] MDL105,519 free

\( a \) = slope \(-(1/K_d)\)

\( x \) = specific \[^3\text{H}\] MDL105,519 bound

\( b \) = X-axis intercept \((B_{\text{max}}/K_d)\)
CHAPTER 3

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CHAPTER THREE: RESULTS

SECTION ONE: CHARACTERISATION OF NMDA RECEPTOR EXPRESSION IN MAMMALIAN CELLS

3.1 SUBCLONING OF NR2D cDNA INTO pcDNA1.1 Amp MAMMALIAN EXPRESSION VECTOR

Mishina and co-workers originally inserted the entire coding sequence of the mouse NR2D subunit between the NcoI and XbaI sites of the Xenopus oocyte expression vector, pSP35T, to generate the pSPGRNR2D construct (Ikeda et al., 1992). Figure 3.1 depicts the restriction map of the pSPGRNR2D plasmid. For efficient expression of the recombinant NR2D subunit in mammalian cells it was necessary to subclone the NR2D cDNA into the pcDNA 1.1 Amp expression vector. Figure 3.2 shows the restriction map of pcDNA 1.1 Amp. The subcloning strategy for NR2D cDNA was as follows (Figure 3.3). The cDNA encoding the full length mouse NR2D subunit was excised at positions -50 bp from the start codon and at +190 bp after the stop codon from the pSPGRNR2D vector using the restriction enzymes Hind III and EcoRI. A fragment of 4.2 kb was generated carrying 5' and 3' cohesive ends. In the same digestion reaction, the pSPGR vector was also digested with PvuI restriction enzyme into two fragments of 1.7 kb and 1.9 kb, to distinguish the insert cDNA from the plasmid. Flat-bed agarose gel electrophoresis was used to separate the DNA fragments. The mammalian expression vector pcDNA 1.1 Amp (4.8 kb) was cleaved with restriction enzymes Hind III and EcoRI. Subsequently, the NR2D cDNA was isolated from an agarose gel by electroelution and the vector DNA was purified using
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phenol/chloroform extraction. The ligation reaction was performed as described in section
2.2.9.5. Competent *E-coli* cells were transformed with the ligation mixture and small-scale
cultures grown overnight. Plasmid DNA was prepared and the positive recombinant
identified by flat-bed agarose gel electrophoresis. The restriction map of pcDNANR2D Amp is illustrated in Figure 3.4. Recombinant pcDNANR2D Amp vector was further verified by restriction enzyme mapping. Figure 3.5 shows the results obtained. Glycerol stocks were prepared of pcDNANR2D Amp plasmid and stored at -80°C until use.

3.2 TRANSIENT EXPRESSION OF RECOMBINANT pcDNANR2D Amp IN HEK 293 CELLS

In initial experiments, pcDNANR2D Amp (10 μg total DNA) was transfected into HEK 293 cells by the calcium phosphate method (section 2.2.13) and cell homogenates were prepared 24 h post-transfection (section 2.2.14.1). Expression of the NR2D subunit was analysed by immunoblotting using 7% SDS-PAGE under reducing conditions with anti-NR2D/2C antibodies (section 2.2.19). Figure 3.6 shows the immunoblot obtained. A single immunoreactive band of M, 150 kDa was detected with anti-NR2D/2C antibodies in immunoblots from cells transfected with pcDNANR2D Amp, which was coincident with that found for adult rat spinal cord membranes. This molecular mass of the immunoreactive species corresponds to the value predicted for the mature polypeptide (140 kDa) derived from the NR2D cDNA sequence, allowing for a contribution by weight for N-glycosylation (Ikeda *et al.*, 1992). The M, 150 kDa immunoreactive band was blocked by prior incubation of anti-NR2D/2C antibodies with the NR2D subunit amino acid sequence used for the
production of the antibody (Figure 3.6). No immunoreactivity was detected in untransfected HEK 293 cells with either anti-NR2D/2C antibodies or with antibody pre-incubated with the respective NR2D subunit peptide (Figure 3.6).

3.3 TRANSIENT CO-EXPRESSION OF NR1-1a AND NR2D SUBUNITS IN HEK 293 CELLS

It has been previously reported that the maximal expression of NR1-1a/NR2A heteromeric receptors in HEK 293 cells as determined by both immunoblotting and single point $[^3]$H MK801 radioligand binding was dependent upon the ratio of the respective DNAs used for the transfection (Cik et al., 1993). Using the established transfection conditions (Cik et al., 1993), the optimum ratio for the maximum expression of NR1-1a/NR2D receptor was investigated by varying the DNA ratios of pCISNR1-1a:pcDNANR2D Amp respectively.

3.3.1 Optimisation of the co-expression of NR1-1a/NR2D heteromeric receptors in HEK 293 cells as determined by immunoblotting

As described above, HEK 293 cells were co-transfected with varying ratios of pCISNR1-1a:pcDNANR2D Amp. The DNA ratios used were 1:1, 1:3 and 1:10 but the total amount of plasmid DNA added per flask was maintained at 10 μg, since earlier studies have shown that this amount of plasmid DNA was optimal for the expression of heteromeric receptors (Cik et al., 1993). Control transfections were carried out in parallel where the DNA ratios of pCISNR1-1a:pCIS were 1:1, 1:3 and 1:10. These control experiments ensured that any
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increase in the level of NR1-1a subunit expression observed was due to the coincident co-expression of the NR2D subunit. Cell homogenates were prepared from the transfected cells and immunoblotting was carried out using anti-NR1-1a (911-920) and anti-NR2D/2C antibodies. Figures 3.7 and 3.8a-b show the immunoblots obtained. In all samples anti-NR1-1a (911-920) antibodies recognised a single polypeptide band with M, 117 kDa. This value agrees with that previously reported for the NR1-1a subunit (Chazot et al., 1992). No immunoreactivity was detected in control cells expressing the NR1-1a subunit alone with anti-NR2D/2C antibodies (Figure 3.7). However, the anti-NR2D/2C antibodies recognised a single immunoreactive band with M, 150 kDa in all samples from cells co-transfected with varying ratios of pCISNR1-1a:pcDNANR2D Amp (Figure 3.8a). The intensity of the M, 150 kDa immunoreactive band correlated well with the amount of NR2D DNA used for the transfections (Figure 3.8a). Samples from cells co-transfected with varying DNA ratios of pCISNR1-1a:pCIS showed a comparable level in the expression of the NR1-1a subunit (Figure 3.7). Similarly, no significant difference in the levels of expression of the NR1-1a subunit was detected from cells co-transfected with pCISNR1-1a:pcDNANR2D Amp DNA ratios of 1:1 and 1:10 (Figure 3.8b). In contrast to these DNA ratios, maximum NR1-1a subunit expression was found with pCISNR1-1a:pcDNANR2D Amp at a DNA ratio of 1:3 (Figure 3.8b).
3.3.2 Optimisation of co-expression of NR1-1a/NR2D heteromeric receptors in HEK 293 cells as determined by [\textsuperscript{3}H] MK801 and [\textsuperscript{3}H] MDL105,519 radioligand binding

Well-washed cell homogenates from HEK 293 cells co-transfected with varying DNA ratios of either pCISNR1-1a:pcDNANR2D Amp, or pCISNR1-1a:pCIS were assayed for [\textsuperscript{3}H] MK801 (20 nM) and [\textsuperscript{3}H] MDL105,519 (1.0 nM) single point radioligand binding activities (sections 2.2.20.2 and 2.2.20.3). The results are shown in Figure 3.9-3.10 and are summarised in Table 3.1. No specific [\textsuperscript{3}H] MK801 radioligand binding was detected to cell homogenates expressing the NR1-1a subunit alone. However, specific [\textsuperscript{3}H] MK801 radioligand binding was found in all samples when the NR1a subunit was co-expressed with the NR2D subunit (Figure 3.9). Specific [\textsuperscript{3}H] MDL105,519 radioligand binding was observed in all samples expressing the NR1-1a subunit alone or in combination with the NR2D subunit (Figure 3.10). No significant difference in the level of specific [\textsuperscript{3}H] MDL105,519 radioligand bound was found in samples expressing the NR1-1a subunit alone. A similar level of specific [\textsuperscript{3}H] MDL105,519 radioligand bound was detected in cells expressing the NR1-1a subunit alone in comparison with cells co-transfected with pCISNR1-1a:pcDNANR2D Amp at a DNA ratio of 1:3 (unpaired Students t-test p<0.05) (Figure 3.10). Specific [\textsuperscript{3}H] MK801 radioligand binding revealed that maximum activity of NR1-1a/NR2D receptors was found at a DNA ratio of 1:3. This DNA ratio gave a \sim 5-8 fold increase in the radioligand binding compared with DNA ratios of 1.1 and 1.10. Similarly, the DNA ratio 1:3 for the NR1-1a/NR2D receptor expressed a 5-8 fold increase of specific [\textsuperscript{3}H] MDL105,519 radioligand bound compared with DNA ratios of 1:1 and 1:10.
In conclusion, the optimum ratio for the maximum expression of NR1-1a/NR2D receptors as determined by both immunoblotting and radioligand binding was at a DNA ratio of 1:3. This ratio was used in all subsequent transfections.

3.4 TRANSIENT CO-EXPRESSION OF NR1-1a AND NR2C SUBUNITS IN HEK 293 CELLS

Previous studies have shown that the optimum ratio for the maximum expression of recombinant NR1-1a/NR2C receptors determined by $[^3]H$ MK801 radioligand binding was achieved using a DNA ratio of 1:10 for the subsequent transfection (Coleman, 1996). To verify this result, the optimum DNA ratio for the maximum expression of NR1-1a/NR2C receptors was investigated. Preliminary control experiments showed that the level of NR1-1a subunit expression was not significantly different in cells co-transfected with varying ratios of pCISNR1-1a:pCIS. Consequently these control assays were not repeated in the NR1-1a/NR2C receptor studies (sections 3.3.1-3.3.2).

3.4.1 Optimisation of co-expression of NR1-1a/NR2C heteromeric receptors in HEK 293 cells as determined by immunoblotting

As above, HEK 293 cells were co-transfected with pCISNR1-1a:pCISNR2C at DNA ratios of 1:1, 1:3 and 1:10 with a total of 10 μg DNA. Cell homogenates were prepared from the transfected cells and the expression of both the NR1-1a and NR2C subunits, were
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demonstrated by immunoblotting using anti-NR1-1a (911-920) and anti-NR2D/2C antibodies. Figure 3.11 shows the immunoblot obtained. In all samples a single immunoreactive band with M, 117 kDa was recognised by anti-NR1-1a (911-920) antibodies (Figure 3.11). The anti-NR2D/2C antibody recognised a single immunoreactive band with M, 145 kDa in all cell homogenates. This value agrees with that previously reported for the NR2C subunit (Chazot et al., 1994). The intensity of the M, 145 kDa immunoreactive band correlated well with the amount of NR2C DNA used for the transfections (Figure 3.11). There was no significant difference in the expression of the NR1-1a subunit of cell homogenates prepared from cells co-transfected with pCISNR1-1a:pCISNR2C at DNA ratios of 1:1 and 1:10. In contrast, a significant increase in the expression of the NR1-1a subunit was found at a DNA ratio of 1:3 (Figure 3.11).

3.4.2 Optimisation of co-expression of NR1-1a/NR2C heteromeric receptors in HEK 293 cells as determined by [H] MK801 and [H] MDL105,519 radioligand binding

Well-washed cell homogenates from HEK 293 cells co-transfected with varying DNA ratios of pCISNR1-1a:pCISNR2C were assayed for [H] MK801 (20 nM) and [H] MDL105,519 (1.0 nM) single point radioligand binding activities. The results are shown in Figure 3.12 and are summarised in Table 3.1. Specific radioligand binding of both [H] MK801 and [H] MDL105,519 was detected in all samples. As for the NR1-1a/NR2D receptor there was no significant difference in the level of [H] MDL105,519 radioligand binding to cells co-transfected with pCISNR1-1a:pCISNR2C at a DNA ratio of 1:3
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compared with cells expressing the NR1-1a subunit alone (Figures 3.10 b and 3.12b) (unpaired Students t-test p<0.05). An increase of 3-4 fold in the level of \(^{3}H\) MK801 radioligand binding activity, was found for the DNA ratio of 1:3 for NR1-1a/NR2C receptors compared with DNA ratios of 1:1 and 1:10 (Figure 3.12a). Similar results were found for single point \(^{3}H\) MDL105,519 radioligand binding activity. Compared with DNA ratios of 1:1 and 1:10 a 5-7 fold increase in the level of \(^{3}H\) MDL105,519 radioligand bound was found for a DNA ratio of 1:3 (Figure 3.12b).

Therefore in summary, in all subsequent transfections for the optimum expression of the NR1-1a/NR2C receptor determined by both immunoblotting and radioligand binding was a DNA ratio of 1:3 was used. This is in direct contrast to that used by Coleman, 1996 i.e. 1:10 DNA ratio for pCISNR1-1a/pCISNR2C.

3.5 SECTION TWO: THE DETERMINATION OF NMDA RECEPTOR-MEDIATED-CELL DEATH POST-TRANSFECTION

3.5.1 The determination of cytotoxicity following the expression of NR1-1a/NR2C and NR1-1a/NR2D heteromeric receptors in HEK 293 cells

Prior studies on recombinant NR1-1a/NR2A receptor expression showed a need for NMDA receptor antagonist protection against cell death (Cik et al., 1993). The cell viability of NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells was compared with untransfected cells and NR1-1a/NR2A receptors cultured in the absence or presence of ketamine (1 mM). Cell viability was quantified using the CytoTox non-radioactive
cytotoxicity assay (2.2.16). The results are summarised in Table 3.2. There was no significant difference in the cell viability of cells expressing NR1-la/NR2C, NR1-la/NR2D receptors, untransfected cells and NR1-la/NR2A receptors cultured in the presence of ketamine post-transfection. However, HEK 293 cells co-transfected with pCISNR1-la/pCISNR2A without ketamine resulted in 27% cell death (Table 3.2).

3.6 SECTION THREE: CHARACTERISATION OF $[^3]$H MDL105,519 RADIOLIGAND BINDING TO NATIVE AND CLONED NMDA RECEPTORS


Initial studies were performed in order to define non-specific $[^3]$H MDL105,519 radioligand binding. The level of $[^3]$H MDL105,519 (1 nM) radioligand binding to untransfected well-washed cell homogenates was assayed in the absence and presence of various glycine site ligands of the NMDA receptor. The glycine site ligands were glycine (1 mM), 7-CLKA (1 mM), GV150,526A (0.1 mM) and GV196,771A (0.1 mM). Figure 3.13 shows the results. Both GV150,526A and GV196,771A displaced the non-specific radioligand binding of $[^3]$H MDL105,519 whereas glycine and 7-CLKA did not affect the level of $[^3]$H MDL105,519 radioligand binding to untransfected cell homogenates. Thereafter, 1 mM glycine was used to define specific binding in all $[^3]$H MDL105,519 radioligand binding assays. In addition $[^3]$H MDL105,519 radioligand bound to glass fiber filters (GF/B) was not displaced by
either glycine (1 mM), GV150,526A (0.1 mM) or GV196,771A (0.1 mM) thus indicating that there was no specific $[^3H]$ MDL105,519 radioligand binding to filters in the presence of glycine, GV150,526A and GV196,771A.

Preliminary kinetic studies were performed by C. Reiss in order to determine the equilibration time of $[^3H]$ MDL105,519 radioligand binding. A time course of specific $[^3H]$ MDL105,519 radioligand binding activity was carried out to adult rat well-washed forebrain membranes (Chazot et al., 1998). Specific $[^3H]$ MDL105,519 radioligand binding activity reached equilibrium after 60 min and remained stable for at least 3 h at 4°C. Both the rate of association and dissociation curves were best fit to a single exponential function, with an association rate constant of $k_{a1} = 3.2 \pm 0.2 \times 10^7$ M$^{-1}$ min$^{-1}$ and a dissociation rate constant of $k_{d1} = 0.080 \pm 0.008$ min$^{-1}$ (Chazot et al., 1998). Since $[^3H]$ MDL105,519 radioligand binding reached equilibrium by 60 min and was constant for 3 h at 4°C, a 90 min incubation period was selected to ensure that a steady state had been attained. The dissociation rate constant determined for $[^3H]$ MDL105,519 radioligand binding to adult rat forebrain membranes was in the right order of magnitude to use a filtration-based protocol for the separation of unbound radioligand (see Appendix).

Therefore in summary, the conditions used for $[^3H]$ MDL105,519 radioligand binding assays to both cloned and native NMDA receptors were an incubation time of 90 min at 4°C and using a rapid filtration-based protocol. Non-specific binding was defined using 1 mM glycine.

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3.6.2 \(^{3}\text{H}\) MDL105,519 SATURATION RADIOLIGAND BINDING ASSAYS

HEK 293 cells were transfected with the appropriate NMDA receptor clones. Well-washed cell homogenates were prepared and assayed for \(^{3}\text{H}\) MDL105,519 radioligand binding activity at 0.2-15 nM concentrations (section 2.2.20.2). Results from the saturation studies were analysed using non-linear least squares regression analysis (section 2.2.21.2). The expression of the respective NMDA receptor subunits was always verified by immunoblotting using the appropriate anti-NMDA receptor subunit specific antibodies.

3.6.2.1 \(^{3}\text{H}\) MDL105,519 saturation radioligand binding to NR1 subunits expressed in HEK 293 cells

As described above, \(^{3}\text{H}\) MDL105,519 saturation radioligand binding to NR1-1a and NR1-2a subunits expressed in HEK 293 cells was performed. Figures 3.14 and 3.15 show the results. Specific \(^{3}\text{H}\) MDL105,519 radioligand binding to both NR1-1a and NR1-2a subunits was saturable and best fit to a one-site hyperbola compared to a two-site model (F-test \(p<0.05\)) (Figures 3.14a and 3.15a). Rosenthal transformation of the saturation data was well approximated with a straight line \((r^2 = 0.8)\) (Figures 3.14b and 3.15b). Tables 3.3 and 3.4 summarise the equilibrium dissociation constants \((K_d)\) and maximal binding \((B_{max})\) values determined from the saturation curves and the Rosenthal transformations. There was no significant difference in the \(K_d\)s or the \(B_{max}\) values determined by either method (unpaired Students t-test \(p<0.05\)). Both NR1-1a \((3.2 \pm 1.9 \text{nM})\) and NR1-2a \((2.7 \pm 2.0 \text{nM})\) exhibited similar affinities for \(^{3}\text{H}\) MDL105,519 (unpaired Students t-test \(p<0.05\)) (Table
3.2) (Chazot et al., 1998). There was no significant difference in the levels of expression of NR1-1a (1.9 ±0.8 pmol/mg protein) and NR1-2a (2.0 ±0.5 pmol/mg protein) subunits in HEK 293 cells (unpaired Students t-test p<0.05) (Table 3.3). At a radioligand concentration equal to the $K_D$s of both splice forms the specific binding was approximately 85% of the total radioligand binding.

3.6.2.2 [3H] MDL105,519 saturation radioligand binding to NR1-1a/NR2 subunit combinations expressed in HEK 293 cells

Heteromeric NMDA receptor combinations i.e., NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D expressed in HEK 293 cells were assayed for [3H] MDL105,519 saturation radioligand binding activity. Figures 3.16-3.19 show the results. Specific [3H] MDL105,519 radioligand binding to all heteromeric NR1-1a/NR2 combinations was saturable and best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05) (Figures 3.16a - 3.19a). Rosenthal transformations of the saturation data were well approximated with a straight line ($r^2 = 0.8-0.9$) (Figures 3.16b - 3.19b). The $K_D$ and $B_{max}$ values determined from either the saturation curve or Rosenthal transformations are summarised in Tables 3.3 and 3.4. There was no significant difference in the $K_D$ or the $B_{max}$ values determined from either analysis (unpaired Students t-test p<0.05). At a radioligand concentration equal to the $K_D$s for the binary NR1-1a/NR2 subtypes the specific binding was approximately 70-85% of the total radioligand binding (Figures 3.16a-3.19a). There was no significant difference in the affinity of [3H] MDL105,519 between the different NR1-1a/NR2 subtype combinations (one-way ANOVA p<0.05) (Tables 3.3 and
Similarly, the Bmax values determined showed that there was no significant difference in the level of NMDA heteromeric receptors expression in HEK 293 cells (one-way ANOVA p<0.05) (Tables 3.3 and 3.4).

3.6.3 [3H] MDL105,519 COMPETITION RADIOLIGAND BINDING ASSAYS

Competition radioligand binding assays were performed by incubating well-washed transfected HEK 293 cell homogenates or adult rat P2 membranes with [3H] MDL105,519 (1 nM) and a range of concentrations of the displacing drug (MDL105,519, GV150,526A or GV196,771A) for 90 min at 4°C (section 2.2.20.1). All displacement assays of [3H] MDL105,519 radioligand binding to NMDA receptors by MDL105,519 were always carried out in parallel with those for GV150,526A and GV196,771A. Results from the radioligand binding studies were analysed using non-linear least squares regression analysis. Competition results were initially fit to a sigmoidal model with a variable slope, the F-test was used to assess whether the one-site or the two-site competition model best fit the data (p < 0.05). For the sigmoidal, one-site and two-site binding models the Kᵢ were calculated using the Cheng-Prusoff equation (section 2.2.21.1) (Cheng and Prusoff, 1973). For all [3H] MDL105,519 radioligand binding studies to NR1-1α/NR2 receptors, the expression of the respective NR2 subunit was always verified by immunoblotting using the appropriate anti-NR2 subunit specific antibody.
3.6.3.1 $[^3]H$ MDL105,519 competition radioligand binding to NR1 subunits expressed in HEK 293 cells by MDL105,519, GV150,526A and GV196,771A

As above, displacement by MDL105,519, GV150,526A and GV196,771A of $[^3]H$ MDL105,519 radioligand binding to both NR1-1a and NR1-2a subunits was performed. Figures 3.20-3.22 show the results. Tables 3.5-3.7 summarise the $K_\text{s}$s and the Hill coefficients ($n_\text{H}$) obtained. Competition binding between $[^3]H$ MDL105,519 and MDL105,519, GV150,526A to both NR1-1a and NR1-2a subunits was best fit to a one-site compared to a two-site model (F-test $p < 0.05$) (Figures 3.20 and 3.21). The $n_\text{H}$s were close to unity and there was no significant difference in $K_\text{s}$s of MDL105,519 and GV150,526A between the different splice forms (unpaired Students t-test $p < 0.05$) (Tables 3.5 and 3.6). In contrast, GV196,771A binding to each of the NR1 splice variants was best fit by a two-site model (F-test $p < 0.05$) (Figure 3.22). The $n_\text{H}$s were significantly $< 1$ indicating the presence of more than one binding site (Table 3.7). The GV196,771A displacement curves were resolved into two sites each present at $\sim 50\%$ with the same apparent $K_\text{s}$s for both NR1-1a and NR1-2a splice forms (Table 3.7).

3.6.3.2 $[^3]H$ MDL105,519 competition radioligand binding to NR1/NR2 subunit combinations expressed in HEK 293 cells by MDL105,519, GV150,526A and GV196,771A

Displacement of $[^3]H$ MDL105,519 radioligand binding to all four NR1-1a/NR2 receptor combinations i.e., NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D
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expressed in HEK 293 cells by MDL105,519, GV150,526A and GV196,771A was carried out. The results are shown in Figures 3.23-3.25. Tables 3.8-3.10 summarise the $K_i$s and $n_H$s obtained. Displacement of $[^3H]$ MDL105,519 radioligand binding to all heteromeric receptors by MDL105,519 was significantly best fit to a one-site model with $n_H$s close to unity (F-test $p < 0.05$) (Figure 3.23). The competition profiles of both GV150,526A and GV196,771A resulted in 100% inhibition curves which were best fit to a two-site compared to a one-site model (F-test $p < 0.05$). The $n_H$s of these inhibition curves were in the range $0.4 - 0.6 \pm 0.2$ (Figures 3.24 and 3.25). There was no significant difference in the affinity of MDL105,519 between the different heteromeric NMDA receptor subtypes (Table 3.8). However, for both GV150,526A and GV196,771A a small but significant selectivity was observed. For the $K_i$ values obtained from a sigmoidal fit, the rank order of decreasing affinity for GV150,526A was NR1-1a/NR2D > NR1-1a/NR2A = NR1-1a/NR2B > NR1-1a/NR2C (Table 3.9). When the curves were analysed by the better fit two-site model, this selectivity was due to a reduced affinity of GV150,526A for the NR1-1a/NR2C high affinity site (Table 3.9). In contrast to GV150,526A, GV196,771A displayed a four-fold lower affinity for NR1-1a/NR2A compared to NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors determined from the sigmoidal competition model (Table 3.10). The $K_i$ values from the two-site model suggest that this is attributable to the low affinity binding component. However, analysis of the curves would suggest that the overall reduced affinity is due to both the high and low sites (Table 3.10). The two sites that were resolved from the inhibition binding of $[^3H]$ MDL105,519 by both GV150,526A and GV196,771A were present in approximately equal proportions (Tables 3.9 and 3.10).
3.6.3.3 $[^3]$H MDL105,519 competition radioligand binding to native membranes by MDL105,519, GV150,526A and GV196,771A

Competition radioligand binding of $[^3]$H MDL105,519 to membranes prepared from adult rat forebrain by GV150,526A, GV196,771A and MDL105,519 were performed. Figure 3.26 shows the displacement curves obtained. The $K_i$s and $n_i$s determined are summarised in Table 3.11. Displacement binding of MDL105,519 was best fit to a one-site compared to a two-site model with $n_i$ close to unity (F-test p<0.05). In contrast, the competition profiles for both GV150,526A and GV196,771A were best fit to a two-site model with $n_i$ s 0.5 ± 0.1 (F-test p<0.05). The $K_i$s for MDL105,519 and GV150,526A were similar to those obtained for heteromeric NR1-1a/NR2A and NR1-1a/NR2B recombinant receptors determined from the sigmoidal model (Tables 3.8, 3.9 and 3.11). However, for GV196,771A the $K_i$ for adult rat forebrain membranes was intermediate between the $K_i$ of NR1-1a/NR2A and NR1-1a/NR2B receptors as determined from the sigmoidal fit (Tables 3.10 and 3.11). As found for the heteromeric recombinant receptors, the proportions of the binding sites resolved by the two-site model was approximately 50% (Tables 3.9, 3.10 and 3.11).
Displacement of $[^3]H$ MDL105,519 radioligand binding to all four NR1-1a/NR2 receptor combinations expressed in HEK 293 cells by both GV150,526A and GV196,771A resulted in inhibition curves which were best fit to a two-site compared to a one-site model (F-test $p<0.05$) (Figures 3.24 and 3.25). Many approaches were used to investigate the delineation of the biphasic nature of the competition curves, the strategies and their respective results are shown in sections 3.7.1-3.7.4.

**3.7.1 DETERMINATION OF PURITY AND STABILITY OF GV150,526A AND GV196,771A AS DETERMINED BY FAST ATOM BOMBARDMENT MASS SPECTROSCOPY**

Biphasic, concave inhibition curves may be the result of several different phenomena which include impurity and/or stability of the displacing ligand. Consequently, the purity and stability of both GV150,526A and GV196,771A was analysed by the technique of fast atom bombardment (FAB) mass spectroscopy by Dr. K. Wellam. A stock solution of either GV150,526A was dissolved in 10 mM KOH and GV196,771A in 1% (v/v) DMSO with subsequent dilutions into 50 mM Tris-citrate, pH 7.4 to a final concentration of 1 mM. An
aliquot (1 ml) was removed from both the solution of GV150,526A and GV196,771A and incubated for 90 min and 4°C. Glycerol was mixed with both the incubated and non-incubated solution of either GV150,526A or GV196,771A and the subsequent mixtures were used for FAB mass spectroscopy. Figures 3.27 and 3.28 show the resultant mass spectra of both GV150,526A and GV196,771A before the incubation. No significant differences were found between the mass spectra of either compounds before or following an incubation for 90 min at 4°C. All molecular entities identified were related to the compounds.

3.7.2 INVESTIGATION OF THE COMPETITION PROFILES FOR THE INHIBITION OF \[^{3}H\] MDL105,519 RADIOLIGAND BINDING TO NR1-1a/NR2A RECEPTORS BY GLYCINE SITE LIGANDS WITH DIVERSE CHEMICAL STRUCTURES

Competition profiles for the inhibition of \[^{3}H\] MDL105,519 radioligand binding to NR1-1a/NR2A receptors expressed in HEK 293 cells by other glycine site ligands with diverse chemical structure including glycine, 5,7 DCKA, L701,324 and L689,560 were investigated. These studies were performed to establish whether these ligands could also resolve more than one binding site, as found for the displacement of \[^{3}H\] MDL105,519 radioligand binding to NR1-1a/NR2 receptors by GV150,526A and GV196,771A. Figure 3.29 shows the results. The \(K_i\)s, and \(n_i\)s obtained are summarised in Table 3.12. Displacement binding of glycine, 5,7 DCKA and L701,324 was best fit to a one-site compared to a two-site model with \(n_i\)s close to unity (F-test \(p < 0.05\)) (Table 3.12). The
displacement curve for L689,560 however, was best fit by a two-site model with \( n_h \) significantly less than one (F-test \( p<0.05 \)) (Table 3.12). It was of interest to study whether inhibition binding of \([^3H]\) MDL105,519 to NR1-1a subunits by L689,560 was best fit to a one-site competition model as for GV150,526A or best fit by a two-site model as for GV196,771A. The displacement curve for the inhibition of \([^3H]\) MDL105,519 radioligand binding to NR1-1a subunits by L689,560 was best fit by a one-site binding model compared to a two-site fit (F-test \( p<0.05 \)) (Figure 3.30). The respective \( K_i \) and \( n_h \) obtained are summarised in Table 3.13. Therefore L689,560 exhibited similar behaviour to GV150,526A with respect to inhibition of binding to single NR1-1a subunits and heteromeric NR1-1a/NR2A receptors.

3.7.3 **ALLOSTERIC MODULATION BY L-GLUTAMATE OF THE BINDING OF GV150,526A AND GV196,771A TO NR1-1a/NR2A EXPRESSED IN HEK293 CELLS AND NATIVE FOREBRAIN MEMBRANES DETERMINED FROM THE COMPETITION RADIOLIGAND BINDING OF \([^3H]\) MDL105,519**

Previous studies have shown that when HEK 293 cells are co-transfected with pCISNR1-1a and pCISNR2A, the expressed receptors are heterogeneous comprising of both unassembled NR1-1a and assembled NR1-1a/NR2A complexes (Chazot *et al.*, 1997b). \([^3H]\) MDL105,519 binds with the same affinity to NR1-1a alone or NR1-1a/NR2A receptors (Chazot *et al.*, 1998). It may be that GV150,526A and possibly, GV196,771A can distinguish between the two NMDA receptor subpopulations, i.e. NR1-1a and NR1-1a/NR2A thus resulting in biphasic displacement curves. Consequently, another strategy
used to address the one versus two-site binding models was to analyse the competition binding of [3H] MDL105,519 to NR1-la/NR2A receptors that is operative only for heteromeric receptors. L-glutamate interacts with assembled NR1-la/NR2A receptors but virtually lacks affinity for unassembled NR1 subunits (Laurie and Seeburg, 1994) since the L-glutamate binding site is localised to the NR2 subunit (Laube et al., 1997). In addition, L-glutamate does not modulate [3H] MDL105,519 radioligand binding to NR1-la subunits or to native membranes (Siegel et al., 1996; Baron et al., 1996). This property of the radioligand is an ideal characteristic to investigate any allosteric influences of L-glutamate on the affinities of both GV150,526A and GV196,771A for heteromeric receptors. Displacement of [3H] MDL105,519 radioligand binding to NR1-la/NR2A receptors expressed in HEK 293 cells, by GV150,526A and GV196,771A ± 10-1000 μM L-glutamate under equilibrium conditions was carried out. The results are shown in Figure 3.31a. There was no significant difference between the inhibition curves of GV150,526A and GV196,771A displacement binding to NR1-la/NR2A receptors ± 100 μM L-glutamate (unpaired Students t-test p < 0.05). Similar results were found for the other L-glutamate concentrations tested (results not shown).

Interestingly, it has been shown that allosteric effects of glutamate were pronounced when measured at different times during ligand association (Grimwood et al., 1993; 1994). At equilibrium any allosteric modulation by L-glutamate on the affinities of both GV150,526A and GV196,771A may be masked as parallel changes in both the association and dissociation rate constants of the ligands may occur in the same direction. Previous reports have shown that L-glutamate allosterically decreases the binding in rat brain membranes of some glycine site antagonists (Grimwood et al., 1992; 1993). Based on these observations
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any allosteric influences of ±100 μM L-glutamate on the competition curves of [³H] MDL105,519 radioligand binding to native membranes by GV150,526A under non-equilibrium conditions was investigated. The results are shown in Figure 3.3.1b. The Kᵢs and nᵢs obtained are summarised in Table 3.14. There was a significant difference between the inhibition curves of GV150,526A displacement binding to adult rat forebrain membranes ± 100 μM L-glutamate. Inhibition binding of [³H] MDL105,519 to adult rat forebrain membranes by GV150,526A +100 μM L-glutamate was best fit to a one-site model compared to a two-site fit (F-test p < 0.05). However, in the absence of added 100 μM L-glutamate, the displacement binding of GV150,526A was best fit to a two-site compared to a one-site model (F-test p< 0.05). In the presence of 100 μM L-glutamate the Kᵢ determined for GV150,526A was 8-fold higher than the Kᵢ calculated in the absence of added L-glutamate (Table 3.14).

3.7.4 [³H] MDL105,519 RADIOLIGAND BINDING TO CELL SURFACE NMDA RECEPTORS EXPRESSED IN HEK 293 CELLS

It has been previously suggested that NR1-1a/NR2A receptors expressed in HEK 293 cells, can exist as a heterogenous population composed of both unassembled NR1-1a and assembled NR1-1a/NR2A complexes (Chazot et al., 1997b). Furthermore, it has been reported that the unassembled NR1-1a subunits may be retained in intracellular occlusions, whereas NR1-1a/NR2A receptor complexes exist at the cell surface (McIlhinney et al., 1998). It may be that both GV150,526A and GV196,771A can distinguish between the two NMDA receptor subpopulations, i.e. NR1-1a and NR1-1a/NR2A thus resulting in biphasic...
displacement curves. These observations led to the approach of cell surface radioligand binding being employed. This technique was used to study the displacement by both GV150,526A and GV196,771A of [³H] MDL105,519 radioligand binding to cell surface receptors rather than to homogenates of transfected cells where putative intracellular pools, would be accessed by the hydrophilic radioligand. Cell surface radioligand binding was performed by transfecting HEK 293 cells with the appropriate NMDA receptor clones. Transfected cells were subjected to a trypsin dissociation 1 h post-transfection and plated onto poly-L-lysine-treated (50 µg/ml) dishes (section 2.2.13.1). Cells were collected 24 h post-transfection and were washed with ice-cold Lockes buffer pH 7.4 and assayed for [³H] MDL105,519 (1 nM) radioligand binding activity for 90 min at 4°C (section 2.2.20.4). A schematic representation of the methodology is depicted in Figure 3.32.

3.7.4.1 OPTIMISATION OF [³H] MDL105,519 RADIOLIGAND BINDING ASSAYS TO CELL SURFACE NMDA RECEPTORS EXPRESSED IN HEK 293 CELLS

3.7.4.1.1 The effect of Lockes buffer on the affinity of MDL105,519, GV150,526A and GV196,771A for NR1-1a/NR2A receptors expressed in HEK 293 cells

Preliminary control experiments were performed to control for the change of buffers from 50 mM Tris-citrate pH 7.1, used in [³H] MDL105,519 radioligand binding to cell homogenates to an isotonic Lockes buffer pH 7.4, employed in HEK 293 cell surface radioligand binding assays. Displacement of [³H] MDL105,519 radioligand binding to well-
washed cell homogenates from HEK 293 cells co-transfected with pCISNR1-1a/pCISNR2A by MDL105,519, GV150,526A and GV196,771A using either 50 mM Tris-citrate pH 7.1, or Lockes buffer pH 7.4 were carried out. The results are shown in Figure 3.33. There was no significant difference between the inhibition curves of MDL105,519, GV150,526A and GV196,771A displacement binding to NR1-1a/NR2A receptors, performed in either 50 mM Tris-citrate pH 7.1 or Lockes buffer pH 7.4 (one-way ANOVA p < 0.05).

3.7.4.1.2 The effect of glycine site ligands on [³H] MDL105,519 radioligand binding to intact untransfected HEK 293 cells

In initial control experiments the effect of various glycine site ligands of the NMDA receptor on the non-specific [³H] MDL105,519 radioligand binding was investigated in order to define non-specific binding in cell surface radioligand binding assays. Untransfected HEK 293 cells were subjected to a trypsin dissociation and were plated onto poly-L-lysine-treated dishes. Cells were washed with ice-cold Lockes buffer pH 7.4, 24 h post-trypsin treatment and incubated with 1 nM [³H] MDL105,519. The level of [³H] MDL105,519 radioligand binding to untransfected intact cells was assayed in the absence and presence of various glycine site ligands including, glycine (1 mM), 7-CLKA (1 mM), 5,7-DCKA (1 mM), GV150,526A (0.1 mM) and GV196,771A (0.1 mM). Figure 3.34 shows the results. There was significant displacement of [³H] MDL105,519 radioligand binding to untransfected intact cells by 7-CLKA, 5,7-DCKA, GV150,526A and GV196,771A. In contrast to these glycine site antagonists, there was no significant
difference in the level of $[^3H]$ MDL105,519 radioligand binding to untransfected intact cells in the presence or absence of 1 mM glycine (unpaired Students t-test $p < 0.05$). Thereafter, 1 mM glycine was used in all subsequent cell surface $[^3H]$ MDL105,519 radioligand binding assays to define non-specific binding.

3.7.4.1.3 Optimisation of the protein concentration for HEK 293 cell surface $[^3H]$MDL105,519 radioligand binding assays

The optimum amount of protein for a high signal to noise ratio of $[^3H]$ MDL105,519 radioligand binding to cell surface receptors was investigated. Increasing amounts of intact HEK 293 cells expressing NR1-1a/NR2A receptor membranes (0.02-0.18 mg protein) was incubated with a fixed concentration of $[^3H]$ MDL105,519 (1 nM). The results are shown in Figure 3.35. The plot of specific $[^3H]$ MDL105,519 radioligand binding to cell surface NR1-1a/NR2A receptors versus amount of protein was linear over the range of 0.02-0.18 mg protein, with a correlation coefficient $r^2 = 0.8$. The y-intercept was zero indicating no specific radioligand binding in the absence of membrane protein. There was a significant increase in the signal to noise ratio of specific $[^3H]$ MDL105,519 radioligand binding to cell surface NR1-1a/NR2A receptors using 0.10 mg compared with 0.18 mg membrane protein (Figure 3.35). Therefore 0.10 mg of protein was used routinely in cell surface radioligand binding assays.
3.7.4.1.4 The effect of trypsin dissociation 1h post-transfection on the expression of both NR1-1a and NR2A subunits expressed in HEK 293 cells

The effect of trypsin-treatment 1 h post-transfection on the expression of both NR1-1a and NR2A subunits was investigated. HEK 293 cells co-transfected with pCISNR1-1a/pCISNR2A were either treated with trypsin 1 h post-transfection or left untreated. Well-washed cell homogenates were prepared from both treated and non-treated cells 24 h post-transfection. The expression of both NR1-1a and NR2A subunits from trypsin and non-treated cell homogenates was analysed by [$^3$H] MDL105,519 radioligand binding activity and immunoblotting. The radioligand binding results are shown in Figure 3.36. In all samples, specific [$^3$H] MDL105,519 radioligand binding was detected. The level of specific [$^3$H] MDL105,519 bound to NR1-1a/NR2A receptors from non-treated control cells was not significantly different to trypsin-treated cells (p<0.005 unpaired Students t-test). These results were further verified by immunoblotting using the appropriate anti-NMDA receptors subunit-specific antibodies. The immunoblots showed that there was no significant difference in the levels of expression of both NR1-1a and NR2A subunits from non-treated and trypsin-treated cells (results not shown). Subsequently, all transfected cells were subjected to a trypsin dissociation 1 h post-transfection.
3.7.4.2 [³H] MDL105,519 RADIOLIGAND BINDING TO CELL SURFACE AND HOMOGENISED MEMBRANES EXPRESSING NR1-1a AND NR1-1a/NR2A RECEPTORS IN HEK 293 CELLS

HEK 293 cells were co-transfected with either pCISNR1-1a/pCIS or pCISNR1-1a/pCISNR2A (10 μg of DNA in a 1:3 ratio). Cells processed as intact cells were subjected to a trypsin dissociation 1 h post-transfection and were plated onto poly-L-lysine-treated dishes. Well-washed cell homogenates or washed intact cells were prepared 24 h post-transfection. Both preparations i.e., cell homogenates and intact cells were assayed for [³H] MDL105,519 radioligand binding activity. Control radioligand binding assays were carried out in parallel with untransfected intact cells. Figure 3.37 shows the results. No specific [³H] MDL105,519 radioligand binding to untransfected intact cells was detected. In contrast, specific [³H] MDL105,519 radioligand binding activity was observed in all transfected samples. There was no significant difference in the levels of specific [³H] MDL105,519 radioligand bound to cell homogenates expressing NR1-1a alone or in combination with the NR2A subunit. The level of specific [³H] MDL105,519 radioligand bound to NR1-1a and NR1-1a/NR2A receptors was approximately 2-3-fold higher for membranes prepared as cell homogenates compared to intact cells (Figure 3.37). Surprisingly, a similar level of specific [³H] MDL105,519 radioligand bound was found to intact cells expressing NR1-1a alone or NR1-1a/NR2A receptors (p<0.005 unpaired Students t-test) (Figure 3.37). The expression of both the NR1-1a and NR2A receptor subunits in intact cells was verified by immunoblotting using the appropriate anti-NMDA receptor subunit-specific antibodies (results not shown).
3.7.5 INVESTIGATION OF EXPRESSION OF NR1-1α SUBUNITS AT THE CELL SURFACE FOLLOWING TRANSFECTION OF HEK 293 CELLS

Previously it has been shown that [$^3$H] MDL105,519 binds to cell surface NR1-1α subunits (3.7.4.2). This finding is in direct conflict with a report that suggests that when the NR1-1α subunit is expressed alone in HEK 293 cells it does not reach the cell surface (McIlhinney et al., 1998). To ascertain that [$^3$H] MDL105,519 radioligand binding occurs to cell surface NR1-1α rather than to intracellular NR1-1α subunit pools, many preliminary control experiments were performed.

3.7.5.1 The effect of trypsin dissociation on the cell viability following the expression of NR1-1α and NR1-1α/NR2A receptors in HEK 293 cells

The effect of trypsin dissociation on the cell viability following the expression of NR1-1α and NR1-1α/NR2A receptors in intact HEK 293 cells was investigated. This determined whether trypsin treatment of cells causes any damage to the plasma membrane, thereby providing [$^3$H] MDL105,519 with access to bind to intracellular NR1-1α subunits. HEK 293 cells co-transfected with either pCISNR1-1α/pCIS or pCISNR1-1α/pCISNR2A were subjected to a trypsin dissociation 1 h post-transfection and were plated onto poly-L-lysine-treated dishes. Control experiments were carried out in parallel with non-trypsin treated cells expressing the NR1-1α subunit and with both trypsin-treated and non-treated untransfected cells. The cell viability of all samples was quantified using the CytoTox non-radioactive cytotoxicity assay. The results are shown in Figure 3.38. There was no
significant difference in the cell viability of trypsin-treated and non-treated untransfected cells, cells expressing the NR1-1a subunit and NR1-1a/NR2A receptors treated with trypsin and cultured in the presence of 1 mM ketamine post-transfection. However, cells expressing NR1-1a/NR2A receptors treated with trypsin 1 h post-transfection and cultured in the absence of ketamine resulted in ~27 % cell death.

3.7.5.2 Inhibition of any uptake of glycine by transporter systems in the membranes of HEK 293 cells

Previous reports show that there is a glycine transporter system present in the membranes of HEK 293 cells (Sato et al., 1995). This observation suggests the possibility that glycine displacement of [³H] MDL105,519 radioligand binding to intact cells expressing NR1-1a subunits could occur at intracellular NR1-1a pools. To elucidate whether this was the case any intracellular glycine uptake was minimised by using a structurally similar ligand to glycine such as L-alanine to compete for the glycine transporter. Preliminary control experiments have shown that [³H] MDL105,519 radioligand binding to well-washed cell homogenates expressing NR1-1a subunits was insensitive to displacement by L-alanine (results not shown). The displacement of [³H] MDL105,519 radioligand binding to intact cells expressing NR1-1a subunits by glycine, in the absence and presence of 5 mM and 50 mM L-alanine was performed. Figure 3.39 show the results obtained. Specific [³H] MDL105,519 radioligand binding was observed in all samples. There was no significant difference in the specific [³H] MDL105,519 radioligand binding to intact cells expressing the NR1-1a subunit, in the absence or presence of 5 mM and 50 mM L-alanine (p<0.05
unpaired Students t-test).

3.7.5.3 Comparison of distribution ratios in octanol/buffer system of $[^3]$H MDL105,519 with hydrophilic and hydrophobic radioligands

There are many criteria that need to be fulfilled in the conditions used for cell surface radioligand binding assays. One such requirement is that the radioligand should be hydrophilic to reduce any equilibration of the ligand across the lipid bilayer of the cell membrane. An index for the hydrophilic nature of $[^3]$H MDL105,519 was defined by determining the distribution coefficients for a range of radioligands in an octanol-buffer system at 4 °C. Table 3.15 summarises the partition coefficients determined. The respective partition coefficient in octanol to buffer for a hydrophilic molecule such as $[^3]$H glycine was 1:230, for a hydrophobic molecule such as $[^3]$H flunitrazepam was 16:1 and for $[^3]$H MDL105,519 was 1:5. The distribution coefficient of $[^3]$H MDL105,519 shows that the radioligand is a hydrophilic compound and would not be expected to transverse the plasma bilayer at 4 °C.

3.7.5.4 Determination of intracellular $[^3]$H MDL105,519 in HEK 293 cells

To elucidate whether there was any equilibration of $[^3]$H MDL105,519 across the cell membrane in cell surface radioligand binding assays, intracellular levels of this radioligand was investigated. Untransfected HEK 293 cells were re-suspended in Locke’s buffer, pH 7.4 and incubated with $[^3]$H MDL105,519 for 90 min at 4 °C. Following the incubation, cells
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were either homogenised to release any intracellular radioligand or left as intact cells. Both intact and homogenised cells were centrifuged and the radioactivity was quantified in the remaining cellular pellets. A schematic representation of the methodology is depicted in Figure 3.40. No significant difference in the levels of [3H] MDL105,519 non-specific radioligand bound to either untransfected cell homogenates or to intact cells was observed (p<0.005 unpaired Students t-test) (Figure 3.41). Thus showing that there was no significant endogenous uptake of the radioligand.

Therefore in summary, both the intact cell surface radioligand binding and control experiments indicate that the NR1-1a subunit may be expressed at the cell surface. Thus the cell surface receptors expressed following the co-transfection in HEK 293 cells with pCISNR1-1a/pCISNR2A may be a heterogeneous population i.e., comprising of both NR1-1a, and NR1-1a/NR2A complexes. Thus clearly these findings invalidate the rationale to use the technique of intact cell radioligand binding to investigate the displacement by both GV150,526A and GV196,771A of [3H] MDL105,519 radioligand binding to cell surface receptors.
Figure 3.1: Schematic representation of the pSPGRNR2D expression vector

The gene for antibiotic resistance (Amp’), restriction polylinker region and site for the production of sense mRNA (SP6) are shown. The entire coding sequence of the mouse NR2D subunit was originally inserted between the Ncol and Xba I sites of the plasmid to generate the pSPGRNR2D expression vector with a size of 7.2 kb (Ikeda et al., 1992). The restriction enzymes Hind III and EcoRI were used to cut the clone at positions -50 bp from the start codon and at +190 bp after the stop codon. A fragment of 4.2 kb was generated carrying 5’ and 3’ cohesive ends.
Figure 3.2: Schematic representation of the pcDNA 1.1 Amp mammalian expression vector

The positions of the bacterial origin of replication (ColE1), gene for antibiotic resistance (Amp'), origin for single strand DNA production (M13), the site for production of sense mRNA (T7), restriction polylinker region, promoter (CMV) and enhancer (SV40) sequences are shown.
Figure 3.3: Subcloning strategy for the insertion of NR2D cDNA into the mammalian expression vector, pcDNA1.1 Amp

The cDNA encoding the full length mouse NR2D subunit was excised from the pSPGR vector using the restriction enzymes HindIII and EcoRI to cut the clone at positions -50 bp from the start codon and at +190 bp after the stop codon. The resulting 4.2 kb fragment was generated and directionally cloned into the HindIII/EcoRI polylinker sites of the pcDNA 1.1 Amp mammalian expression vector to yield recombinant pcDNANR2D Amp plasmid.
The cDNA encoding the full length mouse NR2D subunit (4.2 kb) was directionally cloned into the HindIII/EcoRI polylinker sites of the pcDNA 1.1 Amp mammalian expression vector to yield recombinant pcDNANR2D Amp. The correct orientation of the vector was verified by restriction endonuclease mapping using Cla I, Bcl I and Nhe I.
Figure 3.5: Restriction enzyme mapping of pcDNANR2D Amp vector

Lane 1: Molecular weight standards (kb)
Lane 2: Non-digested pcDNANR2D Amp vector (9.0 kb)
Lane 3: Digestion of the recombinant pcDNANR2D Amp vector with Hind III
Lane 4: pcDNANR2D Amp vector digested with EcoRI
Lane 5: Digestion of the recombinant pcDNANR2D Amp vector with Hind III and EcoRI
Lane 6: Digestion of the recombinant pcDNANR2D Amp vector with Bcl I
Lane 7: Recombinant pcDNANR2D Amp vector cut with Cla I
Lane 8: Recombinant pcDNANR2D Amp vector digested with Nhe I
RESULTS 3

The recombinant pcDNANR2D Amp was digested with selected restriction enzymes to ascertain the correct orientation of the vector. The digested DNA samples were analysed by flat-bed agarose gel electrophoresis with appropriate controls. Lane 2 is the non-digested pcDNANR2D Amp vector (9.0 kb). Recombinant pcDNANR2D Amp vector digested separately with Hind III and EcoR I respectively, produced linearised DNA (lanes 3 and 4). Restriction enzyme digestion with both Hind III and EcoR I gave fragments of 4.2 kb and 4.8 kb which correspond to the NR2D cDNA fragment and the pcDNA vector (lane 5). To characterise the plasmid and insert DNA, specific enzymes were employed in the enzyme digestion i.e. Bcl I was used which cuts the vector and not the insert and Cla I restriction enzyme digests the insert only, the resultant linearised DNA are shown in lanes 6 and 7. DNA fragments of 5.2 kb and 3.8 kb from the Nhe I digestion which cuts both the insert and vector corresponded to the correct size of the recombinant vector, with NR2D cDNA in the correct orientation (lane 8).
Figure 3.6: Immunoblot demonstrating the expression of recombinant NR2D subunit

Membranes were prepared from either HEK 293 cells transfected with pcDNANR2D Amp (10 μg), untransfected cells or adult rat spinal cord. Expression of the NR2D subunit was analysed by immunoblotting using 7% SDS-PAGE under reducing conditions with 50 μg of protein applied per gel lane. Lanes 1-3 were probed anti-NR2D/2C antibodies pre-incubated with the respective NR2D subunit peptide. Lanes 4-6 were blotted with anti-NR2D/2C antibodies. The molecular weight markers (kDa) are shown on the right.

Lane 1: HEK 293 cells homogenates from cells transfected with pcDNANR2D Amp
Lane 2: Membranes from adult rat spinal cord
Lane 3: Untransfected HEK 293 cell homogenates
Lane 4: Untransfected HEK 293 cell homogenates
Lane 5: Membranes from adult rat spinal cord
Lane 6: HEK 293 cells homogenates from cells transfected with pcDNANR2D Amp
Figure 3.7: Immunoblot demonstrating the expression of the NR1-1a subunit following the co-transfection of pCISNR1-1a/pCIS at varying DNA ratios in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1-1a:pCIS at varying DNA ratios of 1:1, 1:3 and 1:10 respectively (10 µg of total DNA). Expression of the NR1-1a subunit was analysed by immunoblotting using 7% SDS-PAGE under reducing conditions with 50 µg of protein applied per gel lane. Affinity-purified antibodies were employed at a final concentration of 1 µg/ml. Lanes 1-3 were probed anti-NR2D/2C antibodies and lanes 5-7 were blotted with anti-NR1-1a (911-920) antibodies. The positions of molecular weight markers (x10^3) are shown on the left.
RESULTS 3

Lanes 1,5: Cell homogenates from cells transfected with pCISNR1-1a :pCIS at a DNA ratio of 1:1

Lanes 2,6: Cell homogenates from cells transfected with pCISNR1-1a :pCIS at a DNA ratio of 1:3

Lanes 3,7: Cell homogenates from cells transfected with pCISNR1-1a :pCIS at a DNA ratio of 1:10
Figure 3.8a: Immunoblot demonstrating the optimum ratio for maximum expression of the NR1-1a/NR2D receptor following the co-transfection of pCISNR1-1a /pcDNANR2D Amp at varying DNA ratios in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1-1a :pcDNANR2D Amp at varying DNA ratios of 1:1, 1:3 and 1:10 respectively. The expression of the NR2D subunit was analysed by immunoblotting using 7 % SDS-PAGE under reducing conditions, with 50 μg of protein applied per gel lane. Affinity-purified antibodies were employed at a final concentration of 1 μg/ml. Lanes 1-3 were probed anti-NR2D/2C antibodies. The positions of molecular weight markers (x10^3) are shown on the left.

**Lane 1:** Cell homogenates from cells transfected with pCISNR1-1a :pcDNANR2D Amp at a DNA ratio of 1:1

**Lane 2:** Cell homogenates from cells transfected with pCISNR1-1a :pcDNANR2D Amp at a DNA ratio of 1:3

**Lane 3:** Cell homogenates from cells transfected with pCISNR1-1a :pcDNANR2D Amp at a DNA ratio of 1:10
Figure 3.8b: Immunoblot demonstrating the optimum ratio for maximum expression of the NR1-1a/NR2D receptor following the co-transfection of pCISNR1-1a /pcDNANR2D Amp at varying DNA ratios in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1-1a :pcDNANR2D Amp at varying DNA ratios of 1:1, 1:3 and 1:10 respectively. The expression of the NR1-1a subunit was analysed by immunoblotting using 7% SDS-PAGE under reducing conditions, with 50 μg of protein applied per gel lane. Affinity-purified antibodies were employed at a final concentration of 1 μg/ml. Lanes 1-3 were probed with anti-NR1-1a (911-920) antibodies. The positions of molecular weight markers (x10^3) are shown on the left.
RESULTS 3

Lane 1: Cell homogenates from cells transfected with pCISNR1-1a : pcDNANR2D Amp at a DNA ratio of 1:1

Lane 2: Cell homogenates from cells transfected with pCISNR1-1a : pcDNANR2D Amp at a DNA ratio of 1:3

Lane 3: Cell homogenates from cells transfected with pCISNR1-1a : pcDNANR2D Amp at a DNA ratio of 1:10
Figure 3.9: Specific $[^3]H$ MK801 radioligand binding to cell homogenates from HEK 293 cells co-transfected with varying DNA ratios of pCISNR1-1a:pcDNANR2D Amp

HEK 293 cells were co-transfected with varying DNA ratios of 1:1, 1:3 and 1:10 of pCISNR1-1a:pcDNANR2D Amp. Cells were collected 24 h post-transfection and well-washed cell homogenates were prepared and assayed for $[^3]H$ MK801 (20 nM) single point radioligand binding activity. Data points are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of $p < 0.05$. 

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Figure 3.10: The effect of varying the pCISNR1-1a:pcDNANR2D Amp and pCISNR1-1a:pCIS DNA ratios used for co-transfection in HEK 293 cells on the specific $^3$H] MDL105,519 radioligand binding to the resultant cell homogenates

HEK 293 cells were co-transfected with varying DNA ratios of 1:1, 1:3 and 1:10 of either A, pCISNR1-1a:pcDNANR2D Amp or B, pCISNR1-1a:pCIS (total 10 µg DNA). Cells were collected 24 h post-transfection and well-washed cell homogenates were prepared and assayed for specific $^3$H]MDL105,519 (1.0 nM) radioligand binding activity. Data points are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of $p < 0.05$. 

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Figure 3.11: Immunoblot demonstrating the optimum ratio for the maximum expression of NR1-1a/NR2C receptor following the co-transfection of pCISNR1-1a:pCISNR2C at varying DNA ratios in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1-1a:pCISNR2C at varying DNA ratios of 1:1, 1:3 and 1:10 respectively. The expression of both the NR1-1a and NR2C subunits was analysed by immunoblotting, using 7 % SDS-PAGE under reducing conditions. Total amount of protein applied per gel lane was 50 μg. Affinity-purified antibodies were employed at a final concentration of 1 μg/ml. Lanes 1-3 were probed anti-NR2D/2C antibodies and lanes 5-7 were blotted with anti-NR1-1a (911-920) antibodies. The positions of molecular weight markers (x10^3) are shown on the left.

**Lanes 1,5:** Cell homogenates from cells transfected with pCISNR1-1a:pCISNR2C at a DNA ratio of 1:1

**Lanes 2,6:** Cell homogenates from cells transfected with pCISNR1-1a:pCISNR2C at a DNA ratio of 1:3

**Lanes 3,7:** Cell homogenates from cells transfected with pCISNR1-1a:pCISNR2C at a DNA ratio of 1:10
Figure 3.12: The effect of varying the pCISNR1-1a:pCISNR2C DNA ratios used for co-transfection in HEK 293 cells on the specific radioligand binding of both $[^3]$H MK801 and $[^3]$H MDL105,519 to the resultant cell homogenates

HEK 293 cells were co-transfected with varying DNA ratios of 1:1, 1:3 and 1:10 of pCISNR1-1a:pCISNR2C. Cells were collected 24 h post-transfection and well-washed cell homogenates were prepared and assayed for either A, $[^3]$H MK801 (20 nM) or B, $[^3]$H MDL105,519 (1.0 nM) single point radioligand binding activities. Data points are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of $p < 0.05$. 

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Table 3.1: A summary of the effect of varying pCISNR1-1a:pcDNANR2D Amp and pCISNR1-1a:pCISNR2C DNA ratios used for co-transfection in HEK 293 cells on the specific [³H] MDL105,519 radioligand binding to the resultant cell homogenates

HEK 293 cells were co-transfected with varying DNA ratios of 1:1, 1:3 and 1:10 of either pCISNR1-1a:pcDNANR2D Amp, or pCISNR1-1a:pCISNR2C (total 10 μg DNA). Well-washed cell homogenates were prepared and assayed for [³H] MDL105,519 (1.0 nM) single point radioligand binding activity. Differences in the ratio of [³H] MDL105,519 radioligand binding activity to NR1-1a/NR2D and NR1-1a/NR2C receptors was expressed with respect to the NR1-1a subunit alone. Data points are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of p< 0.05.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Difference in [³H]MDL105,519 radioligand binding activity (ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a/NR2D 1:1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2D 1:3</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2D 1:10</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2C 1:1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2C 1:3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2C 1:10</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>
RESULTS 3

Table 3.2: The determination of cytotoxicity following the expression of NR1-1a/NR2A, NR1-1a/NR2C and NR1-1a/NR2D heteromeric receptors in HEK 293 cells

HEK 293 cells were co-transfected with either pCISNR1-1a/pCISNR2A, pCISNR1-1a/pCISNR2C or pCISNR1-1a/pcDNANR2D Amp (ratio of 1:3, with a total DNA 10μg). Cells transfected with pCISNR1-1a/pCISNR2A were cultured in the absence and presence of ketamine (1 mM). Cells were collected 20 h post-transfection and cell death was quantified using the CytoTox non-radioactive cytotoxicity assay. The results are expressed with respect to complete LDH release. Values shown are means ± S.D. from three separate experiments carried out on three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of p < 0.05.

<table>
<thead>
<tr>
<th>Transfection Sample</th>
<th>Cell Death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected Cells</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Cells co-transfected with pCISNR1-1a/pCISNR2A</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>Cells co-transfected with pCISNR1-1a/pCISNR2A + ketamine (1 mM)</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>Cells co-transfected with pCISNR1-1a/pCISNR2C</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Cells co-transfected with pCISNR1-1a/pcDNANR2D Amp</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>
RESULTS

Figure 3.13: The effect of glycine site ligands of the NMDA receptor on the $[^3]H$ MDL105,519 radioligand binding to untransfected HEK 293 cells

The level of non-specific $[^3]H$ MDL105,519 (1 nM) radioligand binding to untransfected well-washed cell homogenates was assayed in the absence (total) and presence of various glycine site ligands including glycine (1 mM), 7-CLKA (1 mM), GV150,526A (0.1 mM) and GV196,771A (0.1 mM). Results shown are means ± SD of three separate experiments. Results were analysed using one-way ANOVA with a criterion of significance of $p< 0.05$. 

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Figure 3.14: Saturation isotherm and Rosenthal transformation of $[^3$H]MDL105,519 radioligand binding to NR1-1a subunits expressed in HEK 293 cells

HEK 293 cells were transfected with pCISNR1-1a (10 μg DNA) by the calcium phosphate method. Well-washed cell homogenates were prepared 24 h post-transfection and $[^3$H]MDL105,519 saturation radioligand binding assays were performed. A, is the saturation isotherm showing total (●), non-specific (△) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of $[^3$H]MDL105,519 radioligand binding to NR1-1a splice form was best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05). The Rosenthal transformation was well approximated with a straight line ($r^2 = 0.8$). The $K_D$ and Bmax values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
Figure 3.15: Saturation isotherm and Rosenthal transformation of [\(^3\)H] MDL105,519 radioligand binding to NR1-2a subunits expressed in HEK 293 cells

HEK 293 cells were transfected with pCISNR1-2a (10 µg DNA), by the calcium phosphate method. Well-washed cell homogenates were prepared 24 h post-transfection and [\(^3\)H] MDL105,519 saturation radioligand binding carried out. A, is the saturation isotherm depicting total (■), non-specific (△) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of [\(^3\)H] MDL105,519 radioligand binding to NR1-2a subunits was best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05). The Scatchard transformation was well approximated with a straight line (r\(^2\) = 0.7). The K_D and Bmax values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
Figure 3.16: Saturation isotherm and Rosenthal transformation of \([^3H]\) MDL105,519 radioligand binding to NR1-1a/NR2A receptors expressed in HEK 293 cells

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 µg DNA in a 1:3 ratio) by the calcium phosphate method. Well-washed cell homogenates were prepared and \([^3H]\) MDL105,519 saturation radioligand binding assays carried out. A, is the saturation isotherm showing total (■), non-specific (△) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of \([^3H]\) MDL105,519 radioligand binding to NR1-1a/NR2A receptors was best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05). The Rosenthal transformation was well approximated with a straight line (\(r^2 = 0.8\)). The \(K_D\) and Bmax values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
Figure 3.17: Saturation isotherm and Rosenthal transformation of $[^3H]$ MDL105,519 radioligand binding to NR1-1a/NR2B receptors expressed in HEK 293 cells

$[^3H]$ MDL105,519 saturation radioligand binding to well-washed cell homogenates from HEK 293 cells co-transfected with pCISNR1-1a/pCISNR2B was performed. A, is the saturation isotherm depicting total (●), non-specific (Δ) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of $[^3H]$ MDL105,519 radioligand binding to NR1-1a/NR2B receptors was best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05). The Rosenthal transformation was well approximated with a straight line ($r^2=0.8$). The $K_D$ and Bmax values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
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Figure 3.18: Saturation isotherm and Scatchard transformation of [³H] MDL105,519 radioligand binding to NR1-1a/NR2C receptors expressed in HEK 293 cells

[³H] MDL105,519 saturation radioligand binding to well-washed cell homogenates from HEK 293 cells co-transfected with pCISNR1-1a/pCISNR2C was carried out. A, is the saturation isotherm depicting total (●), non-specific (△) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of [³H] MDL105,519 radioligand binding to NR1-1a/NR2C receptors was best fit to a one-site hyperbola compared to a two-site model (F test p<0.05). The Rosenthal transformation was well approximated with a straight line (r²= 0.9). The K_D and B_max values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
Figure 3.19: Saturation isotherm and Rosenthal transformation of $[^3\text{H}]$ MDL105,519 radioligand binding to NR1-1a/NR2D receptors expressed in HEK 293 cells

$[^3\text{H}]$ MDL105,519 saturation radioligand binding to well-washed cell homogenates from HEK 293 cells co-transfected with pCISNR1-1a/pcDNANR2D Amp was carried out. A, is the saturation isotherm showing total (●), non-specific (△) and specific (○) binding. B, is the Rosenthal transformation of the saturation data. Results shown are from a representative experiment. Saturation curve of $[^3\text{H}]$ MDL105,519 radioligand binding to NR1-1a/NR2D receptors was best fit to a one-site hyperbola compared to a two-site model (F-test p<0.05). The Rosenthal transformation was well approximated with a straight line ($r^2=0.9$). The $K_D$ and $B_{\text{max}}$ values determined from the saturation curve and the Rosenthal transformation are summarised in Tables 3.3 and 3.4.
Table 3.3: A summary of the $K_d$ and $B_{max}$s of $[^3H]$ MDL105,519 radioligand binding to NMDA receptor subunits expressed in HEK 293 cells, determined from the respective saturation isotherms

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a</td>
<td>3.2 ±1.9</td>
<td>1.9 ±0.8</td>
</tr>
<tr>
<td>NR1-2a</td>
<td>2.8 ±2.0</td>
<td>2.0 ±0.5</td>
</tr>
<tr>
<td>NR1-1a/NR2A</td>
<td>1.9 ±1.0</td>
<td>2.3 ±1.6</td>
</tr>
<tr>
<td>NR1-1a/NR2B</td>
<td>3.0 ±2.0</td>
<td>2.2 ±1.5</td>
</tr>
<tr>
<td>NR1-1a/NR2C</td>
<td>1.9 ±0.6</td>
<td>2.9 ±1.9</td>
</tr>
<tr>
<td>NR1-1a/NR2D</td>
<td>2.3 ±0.8</td>
<td>5.5 ±1.5</td>
</tr>
</tbody>
</table>

The $K_d$s and $B_{max}$s were determined from the respective saturation curves shown in Figures 3.14 a-3.19 a. Results are means ± S.D. from three separate experiments from three independent transfections. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p< 0.05$. 

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Table 3.4: A summary of the $K_d$ and $B_{max}$ of $[^3H]$MDL105,519 radioligand binding to NMDA receptor subunits expressed in HEK 293 cells, determined from the respective Rosenthal transformations

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$ (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a</td>
<td>5.0 ±2.2</td>
<td>2.5 ±1.2</td>
</tr>
<tr>
<td>NR1-2a</td>
<td>4.8 ±2.1</td>
<td>2.5 ±0.5</td>
</tr>
<tr>
<td>NR1-1a/NR2A</td>
<td>1.9 ±1.0</td>
<td>1.0 ±0.8</td>
</tr>
<tr>
<td>NR1-1a/NR2B</td>
<td>2.9 ±1.0</td>
<td>1.0 ±0.9</td>
</tr>
<tr>
<td>NR1-1a/NR2C</td>
<td>1.9 ±1.0</td>
<td>1.4 ±0.4</td>
</tr>
<tr>
<td>NR1-1a/NR2D</td>
<td>4.8 ±2.0</td>
<td>7.3 ±4.4</td>
</tr>
</tbody>
</table>

The $K_d$s and $B_{max}$s were determined from the respective Rosenthal transformations shown in Figures 3.14 b-3.19 b. Results are means ± S.D. from three separate experiments from three independent transfections. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p<0.05$. 

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Figure 3.20: Competition profiles for the inhibition of [³H] MDL105,519 radioligand binding to NR1-1a and NR1-2a receptor subunits by MDL105,519

HEK 293 cells were transfected with pCISNR1-1a or pCISNR1-2a (10 μg total DNA) by the calcium phosphate method cells were harvested 24 h post-transfection. Well-washed cell homogenates were prepared and competition of [³H] MDL105,519 radioligand binding to NR1-1a; (○) and NR1-2a; (●) by MDL105,519 performed. Results shown are means ± S.D. for three separate experiments from three independent transfections. Inhibition binding of [³H] MDL105,519 to both NR1 splice forms by MDL105,519 was best fit to a one-site model compared to a two-site fit (F-test p<0.05). The inhibitory constants (K_i) calculated from the IC_{50} values using the Cheng-Prussof equation and the respective Hill coefficients (n_H) are summarised in Table 3.5
Figure 3.21: Competition profiles for the inhibition of $[^3]$H MDL105,519 radioligand binding to NR1-1a and NR1-2a receptor subunits by GV150,526A

Well-washed cell homogenates were prepared from HEK 293 cells transfected with pCISNR1-1a or pCISNR1-2a. Competition of $[^3]$H MDL105,519 radioligand binding to NR1-1a; (○) and NR1-2a; (●) by GV150,526A was performed. Results shown are means ± S.D. for five separate experiments from five independent transfections. Inhibition binding of $[^3]$H MDL105,519 to both the NR1 splice forms by GV150,526A was best fit to a one-site model compared to a two-site fit (F-test p<0.05). The $K_i$s and the respective $n_H$s are summarised in Table 3.6.
Figure 3.22: Competition profiles for the inhibition of $[^3]H$ MDL105,519 radioligand binding to NR1-1a and NR1-2a receptor subunits by GV196,771A

HEK 293 cells were transfected with pCISNR1-1a or pCISNR1-2a (10 μg DNA), by the calcium phosphate method cells were harvested 24 h post-transfection. Well-washed cell homogenates were prepared and competition of $[^3]H$ MDL105,519 radioligand binding to NR1-1a; (○) and NR1-2a; (■) by GV196,771A carried out. Results shown are means ± S.D. for five separate experiments from five independent transfections. Inhibition binding of $[^3]H$ MDL105,519 by GV196,771A to both the NR1 splice forms was best fit to a two-site model compared to a one-site fit (F-test p<0.05). The apparent $K_i$s and the respective $n_H$s are summarised in Table 3.7.
Table 3.5: A summary of the $K_i$ and $n_H$ for MDL105,519 binding to NR1-1a and NR1-2a subunits expressed in HEK 293 cells determined by the displacement of $[^3H]$ MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th></th>
<th>NR1-1a</th>
<th>NR1-2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>1.2 ± 0.9</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>$n_H$</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

The $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.20. Results are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using a two-tailed Students t-test with a criterion of significance of p< 0.05.
Table 3.6: A summary of the $K_i$s and $n_H$ for GV150,526A binding to NR1-1a and NR1-2a subunits expressed in HEK 293 cells determined by the displacement of [$^3$H] MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th></th>
<th>NR1-1a</th>
<th>NR1-2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>3.4 ± 1.5</td>
<td>5.0 ± 3.0</td>
</tr>
<tr>
<td>$n_H$</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

The $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.21. Results are means ± S.D. for five separate experiments from five independent transfections. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p < 0.05$. 
Table 3.7: A summary of the apparent $K_i$s and $n_H$s for GV196,771A binding to NR1-1a and NR1-2a subunits expressed in HEK 293 cells determined by the displacement of [³H] MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th></th>
<th>NR1-1a</th>
<th>NR1-2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (nM)</td>
<td>30 ± 5</td>
<td>39 ± 7</td>
</tr>
<tr>
<td></td>
<td>8 ± 3 (47 ± 2)*</td>
<td>4 ± 2 (48 ± 2)*</td>
</tr>
<tr>
<td></td>
<td>153 ± 41 (53 ± 1)*</td>
<td>121 ± 20 (52 ± 2)*</td>
</tr>
<tr>
<td>$n_H$</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

The $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.22. Results are means ± S.D. for five separate experiments from five independent transfections. *Values are the apparent $K_i$s for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p<0.05$. 

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Figure 3.23: Competition curves for the inhibition of $[^3]$H MDL105,519 radioligand binding by MDL105,519 to NR1-la/NR2A, NR1-la/NR2B, NR1-la/NR2C and NR1-la/NR2D receptors expressed in HEK 293 cells

HEK 293 cells were co-transfected with NMDA receptor clones by the calcium phosphate precipitation method. Cells were harvested 24 h post-transfection and well-washed cell homogenates were prepared. $[^3]$H MDL105,519 radioligand binding competition assays to NR1-la/NR2A (•), NR1-la/NR2B (○), NR1-la/NR2C (△), and NR1-la/NR2D (○) by MDL105,519 were performed. Results shown are means ± S.D. for three separate experiments from three independent transfections inhibition binding of $[^3]$H MDL105,519 by MDL105,519 to all heteromeric subtypes was best fit to a one-site model compared to a two-site fit (F-test p<0.05). The $K_s$ and $n_{i}$s are summarised in Table 3.8.
Figure 3.24: Competition curves for the inhibition of $[^3H]$ MDL105,519 radioligand binding by GV150,526A to NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells.

Competition assays of $[^3H]$ MDL105,519 radioligand binding to NR1-1a/NR2A (■), NR1-1a/NR2B (□), NR1-1a/NR2C (△) and NR1-1a/NR2D (○) heteromeric receptors expressed in HEK 293 cells by GV150,526A were carried out. Results shown are means ± S.D. for five separate experiments from five independent transfections. Inhibition binding of $[^3H]$ MDL105,519 to all heteromeric subtypes by GV150,526A was best fit to a two-site model compared to a one-site fit (F-test p<0.05). The apparent $K_i$s and $n_H$s are summarised in Table 3.9.
Figure 3.25: Competition curves for the inhibition of $[^3]H$ MDL105,519 radioligand binding by GV196,771A to NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells

Competition assays of $[^3]H$ MDL105,519 radioligand binding to NR1-1a/NR2A (■), NR1-1a/NR2B (■), NR1-1a/NR2C (△) and NR1-1a/NR2D (○) heteromeric receptors, expressed in HEK 293 cells by GV196,771A were carried out. Results shown are means ± S.D. for five separate experiments from five independent transfections. Inhibition binding of $[^3]H$ MDL105,519 by GV196,771A to all heteromeric subtypes was best fit to a two-site model compared to a one-site fit (F-test p<0.05). The apparent $K_i$s, and $n_i$s are summarised in Table 3.10.
Table 3.8: A summary of the $K_i$s and $n_H$s for MDL105,519 binding to NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells determined from the inhibition of $[^3H]$MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Recombinant receptor</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a/NR2A</td>
<td>12 ± 4</td>
<td>0.8 ±0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2B</td>
<td>15 ± 5</td>
<td>0.9 ±0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2C</td>
<td>12 ± 6</td>
<td>0.9 ±0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2D</td>
<td>18 ± 7</td>
<td>0.8 ±0.2</td>
</tr>
</tbody>
</table>

The $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.23. Results are means ± S.D. for three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of $p<0.05$. 
Table 3.9: A summary of the apparent $K_i$s and $n_H$s for GV150,526A binding to NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells determined from the inhibition of $[^3H] $MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Recombinant receptor</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a/NR2A</td>
<td>5.9 ± 2.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.7 ± 0.2 (53 ± 2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>84 ±54 (47 ± 2)*</td>
<td></td>
</tr>
<tr>
<td>NR1-1a/NR2B</td>
<td>6.8 ± 2.3</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.5 ±0.1 (56 ± 2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80 ±54 (44 ± 2)*</td>
<td></td>
</tr>
<tr>
<td>NR1-1a/NR2C</td>
<td>14 ± 5</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>3 ±1 (56 ± 2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>109 ±47 (44 ± 2)*</td>
<td></td>
</tr>
<tr>
<td>NR1-1a/NR2D</td>
<td>2.4 ± 1.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.4 ± 0.1 (56 ±2)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>47 ± 25 (44 ±2)*</td>
<td></td>
</tr>
</tbody>
</table>

The apparent $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.24. Results are means ± S.D. for five separate experiments from five independent transfections. *Values are the apparent $K_i$s for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using one-way ANOVA with a criterion of significance of $p<0.05$. 

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Table 3.10: A summary of the apparent $K_s$ and $n_H$ for GV196,771A binding to NR1-1a/NR2A, NR1-1a/NR2B, NR1-1a/NR2C and NR1-1a/NR2D receptors expressed in HEK 293 cells determined from the inhibition of $[^3H]$ MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Recombinant receptor</th>
<th>$K_s$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a/NR2A</td>
<td>157 ± 40</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>6 ± 3</td>
<td>(37 ± 6)*</td>
</tr>
<tr>
<td></td>
<td>484 ± 260</td>
<td>(62 ± 6)*</td>
</tr>
<tr>
<td>NR1-1a/NR2B</td>
<td>35 ± 15</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4 ± 2</td>
<td>(55 ± 4)*</td>
</tr>
<tr>
<td></td>
<td>220 ± 100</td>
<td>(45 ± 5)*</td>
</tr>
<tr>
<td>NR1-1a/NR2C</td>
<td>38 ± 18</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5.1 ± 1</td>
<td>(55 ± 4)*</td>
</tr>
<tr>
<td></td>
<td>180 ± 20</td>
<td>(45 ± 5)*</td>
</tr>
<tr>
<td>NR1-1a/NR2D</td>
<td>38 ± 18</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>4 ± 2</td>
<td>(55 ± 4)*</td>
</tr>
<tr>
<td></td>
<td>153 ± 70</td>
<td>(45 ± 5)*</td>
</tr>
</tbody>
</table>

The apparent $K_s$ were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.25. Results are means ± S.D. for five separate experiments from five independent transfections. *Values are the apparent $K_s$ for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using one-way ANOVA with a criterion of significance of $p < 0.05$. 

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Figure 3.26: Competition curves for the inhibition of \(^3\text{H}\) MDL105,519 radioligand binding by GV150,526A, GV196,771A and MDL105,519 to membranes prepared from adult rat forebrain

Membranes were prepared from adult rat forebrains and \(^3\text{H}\) MDL105,519 radioligand binding competition assays by GV150,526A (■), GV196,771A (▲) and MDL105,519 (○) carried out. Results shown are means ± S.D. for three separate experiments. Inhibition binding of \(^3\text{H}\) MDL105,519 to adult rat forebrain membranes by MDL105,519 was best fit to a one-site model compared to a two-site fit (F-test \(p<0.05\)). In contrast to MDL105,519, the inhibition binding of \(^3\text{H}\) MDL105,519 by both GV150,526A and GV196,771A was best fit to a two-site model compared to a one-site fit (F-test \(p<0.05\)). The apparent \(K_i\)s and \(n_H\)s are summarised in Table 3.11.
Table 3.11: A summary of the apparent $K_i$s and $n_H$s for GV150,526A, GV196,771A, and MDL105,519 binding to adult rat forebrain membranes determined by the displacement of $[^3H]$ MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Glycine site antagonist</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDL105,519</td>
<td>15 ± 10</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>GV150,526A</td>
<td>7.5 ± 4.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2.6 ± 1.2 (51 ± 3)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42 ± 34   (49 ± 3)*</td>
<td></td>
</tr>
<tr>
<td>GV196,771A</td>
<td>95 ± 10</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>9.2 ± 7.2 (44 ± 5)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 ± 150 (56 ± 6)*</td>
<td></td>
</tr>
</tbody>
</table>

The apparent $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.26. Results are means ± S.D. for three separate experiments. *Values are the apparent $K_i$s for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using one-way ANOVA with a criterion of significance of p< 0.05.
A stock solution of GV150,526A was dissolved in 10 mM KOH with subsequent dilutions into 50 mM Tris-citrate, pH 7.4 to a final concentration of 1 mM. An aliquot (1 ml) was removed from the solution of GV150,526A and was mixed with glycerol. The sample was analysed by the technique of FAB mass spectroscopy. The resultant mass spectra is shown along with the identify of each molecular entity. The insert shows the structure of GV150,526A as a sodium salt, with a molecular weight of 396 which is denoted M in the Figure.
RESULTS 3

\[ M = 396 \]

\[ [M + (H^+)]^+ \quad 397 \]

\[ [M - (Na^+) + (3H^+)]^+ \quad 282 \]

\[ [M - (Na^+, C_6H_5NH_3) + (2H^+)]^+ \quad 375 \]

\[ [M + (Na^+)]^+ \quad 419 \]
Figure 3.28: FAB mass spectra of GV196,771A

A stock solution of GV196,771A was dissolved in 1% (v/v) DMSO with subsequent dilutions into 50 mM Tris-citrate, pH 7.4 to a final concentration of 1 mM. An aliquot (1 ml) was removed from the solution of GV196,771A and was mixed with glycerol. The sample was analysed by FAB mass spectroscopy. The resultant mass spectra is shown along with the identify of each molecular entity. The insert shows the structure of GV196,771A with a molecular weight of 400 which is denoted M in the Figure.
RESULTS

401
[M + (H\(^+\))\(^+\)]

357
[M - (CO\(_2\)H) + (2H\(^+\))]\(^+\)

365
[M - (HCl) + (H\(^+\))]\(^+\)

329
[M - (2HCl) + (H\(^+\))]\(^+\)

423
[M + (Na\(^+\))]\(^+\)

38E6
3.6E6
3.4E6
3.2E6
3.0E6
2.8E6
2.6E6
2.4E6
2.3E6
2.1E6
1.9E6
1.7E6
1.5E6
1.3E6
1.1E6
9.4E5
7.5E5
5.6E5
3.8E5
1.9E5
0.0E0

m/z

0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100

240 260 280 300 320 340 360 380 400 420 440 460 480 500 520 540 560 580 600
Figure 3.29: Competition curves for the inhibition of $[^3]H$ MDL105,519 radioligand binding to NR1-1a/NR2A receptors expressed in HEK 293 cells by glycine, 5,7 DCKA, L701,324 and L689,560

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 μg of DNA in a 1:3 ratio) by the calcium phosphate precipitation method. Cells were harvested 24 h post-transfection, well-washed cell homogenates prepared and $[^3]H$ MDL105,519 radioligand binding competition assays carried out. Data points are means ± S.D. for three separate experiments from three independent transfections. For glycine (□), 5,7 DCKA (●) and L701,324 (○) inhibition of $[^3]H$ MDL 105,519 radioligand binding was best fit to a one-site model, whereas L689,560 (■) was best fit by a two-site compared to a one-site model (F-test p<0.05). The apparent $K_i$s and $n_H$s are summarised in Table 3.12.
Table 3.12: A summary of the apparent $K_i$s and $n_H$s for glycine, 5,7 DCKA, L701,324 and L689,560 binding to NR1-1a/NR2A receptors expressed in HEK 293 cells determined from the inhibition of $[^3H]$ MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Glycine site ligand</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>3900 ± 1000</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>5,7 DCKA</td>
<td>50 ± 10</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>L701,324</td>
<td>4.2 ± 1.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>L689,560</td>
<td>6.0 ± 1.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.4 ± 0.7 (46 ± 4)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 ± 43 (54 ± 4)*</td>
<td></td>
</tr>
</tbody>
</table>

The apparent $K_i$s were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.29. Results shown are means ± S.D. for three separate experiments from three independent transfections. *Values are the apparent $K_i$s for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using one-way ANOVA with a criterion of significance of $p<0.05$. 

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Figure 3.30: Competition curves for the inhibition of \[^{3}H\] MDL105,519 radioligand binding to NR1-1a and NR1-1a/NR2A receptors expressed in HEK 293 cells by L689,560

HEK 293 cells were transfected with either pCISNR1-1a (10 µg of total DNA), or pCISNR1-1a/pCISNR2A (10 µg of total DNA, in 1:3 ratio) by the calcium phosphate precipitation method. Well-washed cell homogenates were prepared 24 h post-transfection, and \[^{3}H\] MDL105,519 radioligand binding competition assays carried out. Results shown are means ± S.D. for four separate experiments from four independent transfections. L689,560 inhibition of \[^{3}H\] MDL105,519 radioligand binding to NR1-1a (■) subunits was best fit by a one-site model, whereas inhibition binding of \[^{3}H\] MDL105,519 to NR1-1a/NR2A (▲) receptors by L689,560 was best fit to a two-site model (F-test p<0.05). The apparent K_s and n_Hs are summarised in Table 3.13.
Table 3.13: A summary of the \( K_s \) and \( n_H \) for L689,560 binding to NR1-1a and NR1-1a/NR2A receptors expressed in HEK 293 cells determined by the inhibition of \([^3H]\) MDL105,519 radioligand binding

<table>
<thead>
<tr>
<th>Recombinant subtype</th>
<th>( K_I ) (nM)</th>
<th>( n_H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1-1a</td>
<td>7 ± 4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>NR1-1a/NR2A</td>
<td>6.0 ± 1.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.4 ± 0.7 (46 ± 4)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70 ± 43 (54 ± 4)*</td>
<td></td>
</tr>
</tbody>
</table>

The apparent \( K_s \)s were calculated using the Cheng-Prussof equation with the IC\(_{50}\) values determined from the respective displacement curves shown in Figure 3.30. Results are means ± S.D. for four separate experiments from four independent transfections. *Values are the apparent \( K_s \)s for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using a two-tailed Students t-test with a criterion of significance of \( p<0.05 \).
RESULTS 3

Figure 3.31a: The effect of L-glutamate on the competition curves for the inhibition of [³H]MDL105,519 radioligand binding to NR1-1a/NR2A receptors expressed in HEK 293 cells by GV150,526A and GV196,771A under equilibrium conditions

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 µg of DNA in a 1:3 ratio) by the calcium phosphate precipitation method. Cells were harvested 24 h post-transfection, well-washed cell homogenates prepared and [³H]MDL105,519 radioligand binding competition assays carried out ± 100 µM L-glutamate under equilibrium conditions. A, displacement by GV150,526A; B, displacement by GV196,771A; control (○); + 100 µM L-glutamate (●). Results shown are means ± S.D. from three separate experiments from three independent transfections. Inhibition binding of [³H]MDL105,519 by GV150,526A and GV196,771A to NR1-1a/NR2A receptors ± 100 µM L-glutamate was best fit to a two-site model compared to a one-site fit (F-test p<0.05).
RESULTS
Figure 3.31b: The effect of L-glutamate on the competition curve for the inhibition of [3H] MDL105,519 radioligand binding to adult rat forebrain membranes by GV150,526A under non-equilibrium conditions.

[3H] MDL105,519 radioligand binding competition assays to adult rat forebrain membranes by GV150,526A ± 100 μM L-glutamate under non-equilibrium conditions was performed. Inhibition binding of [3H] MDL105,519 by GV150,526A to adult rat forebrain membranes -100 μM L-glutamate was best fit to a two-site model compared to a one-site fit (unpaired Students t-test p<0.05). However, in the presence 100 μM L-glutamate the inhibition binding of [3H] MDL105,519 by GV150,526A was best fit to a one-site compared to a two-site model (unpaired Students t-test p<0.05). The apparent K_i's and n_i's are summarised in Table 3.14.
Table 3.14: A summary of the effect of L-glutamate on the apparent $K_s$ and $n_H$ for the inhibition of $[^3H]$ MDL105,519 radioligand binding by GV150,526A to adult rat forebrain membranes under non-equilibrium conditions

<table>
<thead>
<tr>
<th>Displacement Conditions</th>
<th>$K_i$ (nM)</th>
<th>$n_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV150,526A</td>
<td>10</td>
<td>0.6</td>
</tr>
<tr>
<td>-100 $\mu$M L-glutamate</td>
<td>0.4 (26)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (74)*</td>
<td></td>
</tr>
<tr>
<td>GV150,526A</td>
<td>75</td>
<td>0.9</td>
</tr>
<tr>
<td>+100 $\mu$M L-glutamate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The $K_s$ were calculated using the Cheng-Prussof equation with the IC$_{50}$ values determined from the respective displacement curves shown in Figure 3.31b. Results are from two separate experiments. *Values are the apparent $K_s$ for the two-site binding model with the percentage contribution for each site given in brackets. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p<0.05$. 

-195-
HEK 293 cells were transfected with the appropriate NMDA receptor clones by the calcium phosphate precipitation method. Cells were treated with trypsin 1 h post-transfection and plated onto poly-L-lysine-treated (50 μg/ml) dishes. Cells were washed with ice-cold Lockes buffer 24 h post-trypsin dissociation and assayed for [³H] MDL105,519 radioligand binding activity for 90 min at 4°C.
Figure 3.33: The effect of Lockes buffer on the competition curves for the inhibition of $[^3H]$ MDL105,519 radioligand binding by MDL105,519, GV150,526A and GV196,771A to NR1-1a/NR2A receptors expressed in HEK 293 cells

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 μg of total DNA in a 1:3) by the calcium phosphate precipitation method. Cells were harvested 24 h post-transfection and well-washed cell homogenates prepared. Competition assays of $[^3H]$ MDL105,519 radioligand binding to NR1-1a/NR2A receptors by A, MDL105,519, B, GV150,526A and C, GV196,771A were carried out in either 50 mM Tris-citrate pH 7.1; (■) or Lockes buffer pH 7.4; (○). Results are means ± S.D. from a representative experiment which was repeated twice from two independent transfections.
Figure 3.34: The effect of glycine site ligands of the NMDA receptor on the radioligand binding of [³H] MDL105,519 to untransfected intact HEK 293 cells

Untransfected HEK 293 cells were subjected to a trypsin dissociation and were plated onto poly-L-lysine-treated dishes. Cells were washed 24 h post-trypsin dissociation with ice-cold Lockes buffer pH 7.4 and incubated with 1 nM [³H] MDL105,519. The level of non-specific [³H] MDL105,519 radioligand binding to untransfected intact cells was assayed in the absence (total) and presence of various glycine site ligands including glycine (1 mM), 7-CLKA (1 mM), 5,7-DCKA (1 mM), GV150,526A (0.1 mM) and GV196,771A (0.1 mM). Results shown are means ± S.D. of three separate experiments. Results were analysed using one-way ANOVA with a criterion of significance of p < 0.05.
Figure 3.35: Optimisation of the protein concentration for $[^3]$H MDL105,519 radioligand binding to intact HEK 293 cells expressing NR1-1a/NR2A receptors

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 μg of total DNA in a 1:3) by the calcium phosphate precipitation method. Cells were subjected to a trypsin dissociation 1 h post-transfection and were plated onto poly-L-lysine-treated dishes with varying amounts of protein (0.02-0.18 mg). Well-washed intact cells were assayed for $[^3]$H MDL105,519 (1 nM) radioligand binding activity 24 h post-transfection for 90 min at 4 °C. The plot of specific $[^3]$H MDL105,519 radioligand binding to cell surface NR1-1a/NR2A receptors versus amount of protein was fit by linear regression ($r^2 = 0.8$). Results are means ± S.D. of three separate experiments from three independent transfections.
Figure 3.36: The effect of trypsin-treatment on the level of [$^3$H] MDL105,519 radioligand binding to NR1-1a/NR2A receptors expressed in HEK 293 cells

HEK 293 cells were co-transfected with pCISNR1-1a/pCISNR2A (10 μg of total DNA in a 1:3 ratio) by the calcium phosphate precipitation method. Transfected cells were subjected to a trypsin dissociation 1 h post-transfection and were plated onto poly-L-lysine-treated dishes. Control cells expressing NR1-1a/NR2A receptors were not subjected to a trypsin dissociation. Well-washed cell homogenates from trypsin-treated and control cells were prepared 24 h post-transfection and assayed for [$^3$H] MDL105,519 radioligand binding activity. Results are means ± S.D. Three separate experiments are shown from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of p< 0.05.
RESULTS 3

Figure 3.37: Comparison of specific $[^3]H$ MDL105,519 radioligand bound to NR1-1a and NR1-1a/NR2A receptors expressed in HEK 293 cells from membranes prepared as cell homogenates or intact cells

HEK 293 cells were co-transfected with either pCISNR1-1a/pCIS or pCISNR1-1a/pCISNR2A (10 μg of total DNA in a 1:3 ratio) by the calcium phosphate precipitation method. Cells processed as intact cells were subjected to a trypsin dissociation 1 h post-transfection and were plated onto poly-L-lysine-treated dishes. Cells were collected 24 h post-transfection and either well-washed cell homogenates or intact cells were prepared. Both membrane preparations were assayed for $[^3]H$ MDL105,519 (1 nM) radioligand binding activity. Results shown are means ± S.D. from a representative experiment which was repeated six times from six independent transfections. Results were analysed using a two-tailed Students t-test with a criterion of significance of $p<0.05$. 
RESULTS 3

Homogenised cells expressing NRI-1a/NR2A receptors
Homogenised cells expressing NRI-1a subunits
Intact cells expressing NRI-1a/NR2A receptors
Intact cells expressing NRI-1a subunits

Membrane Preparation

Specific Bound
$[^3]H$ MDL 105,519 (10^-3 mol/mg protein)
RESULTS

Figure 3.38: The effect of trypsin dissociation on cell death following the expression of NR1-1a and NR1-1a/NR2A receptors in HEK 293 cells

HEK 293 cells were co-transfected with either pCISNR1-1a/pCIS or pCISNR1-1a/pCISNR2A (10 μg of total DNA in a 1:3 ratio) by the calcium phosphate precipitation method. Transfected and untransfected cells were subjected to a trypsin dissociation and were plated onto poly-L-lysine-treated culture dishes. Cells co-transfected with pCISNR1-1a/pCISNR2A were cultured in the absence or presence of ketamine (1 mM). Control untransfected cells and cells expressing the NR1-1a subunit were not treated with trypsin. Cell viability of both non-treated and trypsin-treated cells was quantified using the CytoTox non-radioactive cytotoxicity assay. The results are expressed with respect to maximum LDH release. Values shown are means ± S.D. of three separate experiments performed on three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of p< 0.05.
RESULTS 3

![Graph showing cell death percentages for different samples.](image-url)

- Non-treated untransfected cells
- Trypsin-treated untransfected cells
- Non-treated cells expressing NRI-1a
- Trypsin-treated cells expressing NRI-1a
- Trypsin-treated cells expressing NRI-1a/NR2A +ketamine
- Trypsin-treated cells expressing NRI-1a/NR2A +ketafine

Cell Death (%) vs. Samples
Figure 3.39: Comparison of the displacement [³H] MDL105,519 radioligand binding to intact HEK 293 cells expressing NR1-1a subunits by glycine in the absence or presence of L-alanine.

HEK 293 cells were transfected with pCISNR1-1a (10 µg total DNA) by the calcium phosphate precipitation method. Cells were treated with trypsin 1 h post-transfection, and plated onto poly-L-lysine-treated dishes. Well-washed intact cells were prepared 24 h post-transfection. Displacement of [³H] MDL105,519 (1 nM) radioligand binding to intact cells expressing NR1-1a subunits by glycine in the absence and presence of 5 and 50 mM L-alanine was performed. Values shown are means ± S.D. from three separate experiments from three independent transfections. Results were analysed using one-way ANOVA with a criterion of significance of p< 0.05.
Table 3.15: Comparison of the distribution ratios in an octanol-buffer system of $[^3\text{H}]$ MDL105,519 with hydrophilic and hydrophobic radioligands

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>K (Octanol : Buffer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3\text{H}]$ Glycine</td>
<td>1: 230</td>
</tr>
<tr>
<td>$[^3\text{H}]$ Flunitrazepam</td>
<td>16 : 1</td>
</tr>
<tr>
<td>$[^3\text{H}]$ MDL105,519</td>
<td>1: 5</td>
</tr>
</tbody>
</table>

Radioligands (1 nM) were dissolved in Lockes buffer and were thoroughly shaken with an equal volume of octanol. Following a 90 min incubation at 4 °C an aliquot from each phase was collected and the radioactivity was quantified. The distribution coefficient, K, was calculated from the following equation using the concentrations of the radioligand in each phase.

$$
\frac{[^3\text{H}] \text{ Radioligand}}{\text{Octanol}} = K \frac{[^3\text{H}] \text{ Radioligand}}{\text{Buffer}}
$$

The values shown are from a representative experiment which was repeated three times.
Untransfected HEK 293 cells were suspended in Lockes buffer, pH 7.4 and incubated with \[^3\text{H}\] MDL105,519 (1 nM) for 90 min at 4°C. Following the incubation, cells were either homogenised to release any intracellular radioligand or left as intact cells. Both intact and homogenised cells were centrifugated and the supernatant was discarded. The radioactivity in the remaining cellular pellets was quantified.
Figure 3.41: Comparison of the level of $[^3]$H MDL105,519 non-specific radioligand bound to either untransfected cell homogenates or intact cells

Untransfected HEK 293 cells were re-suspended in Locke's buffer, pH 7.4 and incubated with $[^3]$H MDL105,519 for 90 min at 4°C. Following the incubation, cells were either homogenised to release any intracellular radioligand or left as intact cells. Both intact and homogenised cells were centrifuged and the $[^3]$H MDL105,519 non-specific radioligand bound to either untransfected cell homogenates or to intact cells was quantified in the remaining cellular pellets. Values shown are means ± S.D. from two separate experiments. Results were analysed using a two-tailed Students t-test with a criterion of significance of p< 0.05.
CHAPTER FOUR: DISCUSSION

4.0 SECTION ONE: CHARACTERISATION OF NMDA RECEPTOR EXPRESSION IN MAMMALIAN CELLS

The focus of this PhD thesis was to study the binding properties and NMDA receptor subtype-selectivity of three glycine site antagonists, MDL105,519, GV150,526A and GV196,771A to both native and cloned NMDA receptor subtypes using radioligand binding. To elucidate the binding properties of these antagonists to cloned receptor subunits, individual receptor subunit cDNAs were expressed in an in vitro system. A mammalian cell transient expression system was used as it permits post-translational modification of the expressed protein. HEK 293 cells were employed for the transfections as these cells do not express the endogenous genes of interest and are well characterised for high expression of ion channel receptors (e.g. Werner et al., 1991).

In the co-transfection studies, NMDA receptor cDNAs used were from two different species; NR1-1a was a rat cDNA and mouse cDNAs encoded the NR2 subunits. The predicted amino acid sequences for the mature polypeptides of the rat NR1 subunits have ~99.8% amino acid sequence identity with the mouse counterparts, whereas the rat and mouse NR2 protein sequences are more diverse. Therefore the heteromeric receptors i.e., NR1-1a/NR2 can be considered as mouse NMDA receptors.
4.1 SUBCLONING OF NR2D cDNA INTO pcDNA1.1 Amp MAMMALIAN EXPRESSION VECTOR AND THE TRANSIENT EXPRESSION OF pcDNANR2D Amp IN HEK 293 CELLS

Prior to the transient expression of the NR2D subunit in HEK 293 cells, it was necessary to subclone the NR2D cDNA from the *Xenopus* pSP35T plasmid into the pcDNA1.1 Amp mammalian expression vector. It has been reported that untranslated 5' and 3' ends of the NMDA receptor clones inhibit the levels of the respective subunit expression in mammalian cells (Wood *et al.*, 1996). This latter factor influenced the design of the subcloning strategy. Both *Hind III* and *EcoRI* were used to excise the mouse NR2D cDNA at positions -50 bp from the start codon and at +190 bp after the stop codon from the pSPGRNR2D vector. This digestion resulted in the reduction in any 5' and 3' non-coding DNA of the NMDA receptor subunit clone.

The choice of the mammalian expression vector to be employed in the ligation reactions was dependent on many considerations. Firstly, the expression vector should be driven by the cytomegalovirus (CMV) promoter region which is compatible with HEK 293 cells. Secondly, the plasmid required the correct orientation of the restriction sites in the polylinker site, i.e. *Hind III* and *EcoRI* to allow directional cloning of the NR2D subunit cDNA insert. The mammalian expression vector, pcDNA1.1 Amp, fulfilled these criteria. Following the successful ligation of the NR2D subunit into the pcDNA1.1 Amp plasmid, the expression of the subunit in HEK 293 cells was verified by immunoblotting using anti-NR2D/2C antibodies. A single immunoreactive band of M, 150 kDa was detected with anti-
NR2D/2C antibodies (Figure 3.6). This molecular mass of the immunoreactive species corresponds to the value predicted for the mature polypeptide (140 kDa) derived from the NR2D cDNA sequence allowing for a contribution by weight for N-glycosylation (Ikeda et al., 1992). Similar results were reported with polyclonal anti-NR2D antibodies recognising immunoreactive species of M, 150 kDa in the adult and developing rat brain (Wenzel et al., 1996).

4.2 TRANSIENT CO-EXPRESSION OF NR1-1a/NR2C AND NR1-1a/NR2D HETEROMERIC RECEPTORS IN HEK 293 CELLS

It has been previously reported that the maximal expression of NR1-1a/NR2A heteromeric receptors in HEK 293 cells as determined by both immunoblotting and single point [\(^3\)H] MK801 radioligand binding was dependent upon the ratio of the respective DNAs used for the transfection (Cik et al., 1993). Using the established transfection conditions (Cik et al., 1993), the optimum ratio for the maximum expression of NR1-1a/NR2C and NR1-1a/NR2D receptors was investigated by varying the DNA ratios of pCISNR1-1a:pCISNR2C and pCISNR1-1a:pcDNANR2D Amp respectively. The expression of the heteromeric receptors was analysed by immunoblotting, [\(^3\)H] MK801 and [\(^3\)H] MDL105,519 single point radioligand binding activities.

Immunoblotting confirmed the expression of the NR1-1a, NR2C and NR2D subunits in transfected HEK 293 cells for all the DNA ratios investigated. Specific [\(^3\)H] MDL105,519 radioligand binding was also observed in all samples expressing the NR1-1a subunit alone.
or in combination with the NR2C or NR2D subunits (Figure 3.10). These results confirm the findings that the NR1 subunit expressed alone in mammalian cells contains the determinants for glycine site antagonist binding. However, no specific $[^3]H$ MK801 radioligand binding was detected to cell homogenates expressing the NR1-1a subunit alone. In contrast, specific $[^3]H$ MK801 radioligand binding was found in all samples when the NR1a subunit was co-expressed with either the NR2C or NR2D subunits. These findings are consistent with reports that have shown that the NR1 subunit alone is not sufficient for reconstitution of the $[^3]H$ MK801 binding site (Ishmael et al., 1996). Expression studies of the different NMDA receptor subunits in heterologous systems have concluded that highly active NMDA receptor channels are formed from the co-assembly of both an NR1 and NR2 subunit (Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). However, site-directed mutagenesis data suggest that the NR1 subunit is still likely to make an essential contribution to non-competitive antagonist binding domains within the NMDA receptor channel (Burnashev et al., 1992a; Sakurada et al., 1993). Failure to detect a high affinity $[^3]H$ MK801 radioligand binding to NR1-1a subunits is in direct contrast to reports of $[^3]H$ MK801 radioligand binding in membranes derived from HEK 293 cells transiently transfected with the NR1-1a clone (Chazot et al., 1992; Laurie and Seeburg, 1994). Differences in these results are likely to be explained by variations in the radioligand binding procedure including the definition of non-specific binding. Both Chazot et al., (1992) and Laurie and Seeburg, (1994) used unlabelled MK801 to define specific binding in $[^3]H$ MK801 radioligand binding assays. Preliminary results have shown that MK801 competes with $[^3]H$ MK801 radioligand binding to untransfected HEK 293 cells, therefore
both Chazot et al., (1992) and Laurie and Seeburg, (1994) could have characterised non-specific \(^{3}\text{H}\) MK801 radioligand binding (results not shown). In this study, a structural dissimilar ligand to MK801, TCP, was used to define specific \(^{3}\text{H}\) MK801 radioligand binding. The latter compound does not compete with non-specific \(^{3}\text{H}\) MK801 radioligand binding to untransfected HEK 293 cells (results not shown).

The co-assembly of the NR1-1a with either the NR2C or NR2D subunits has been demonstrated by immunoprecipitation (Chazot et al., 1994; Dunah et al., 1998). The functionality of NR1-1a/NR2C receptors was shown by L-glutamate-activated \(\text{Ca}^{2+}\) influx (Grant et al., 1997). Similarly, functionality of NR1-1a/NR2D receptors has been addressed. Buller et al., (1997) have demonstrated that the co-expression of both NR1-1a and NR2D in \textit{Xenopus} oocytes elicits responses to NMDA and glycine.

Specific \(^{3}\text{H}\) MK801, \(^{3}\text{H}\) MDL105,519 radioligand binding and immunoblotting together revealed that maximum activity of both NR1-1a/NR2C and NR1-1a/NR2D receptors was found at a DNA ratio of 1:3. This optimum ratio may be a reflection on differences in efficiency of transcription, translation and protein stability for both NR1-1a and NR2 receptor subunits or the subunit stiochiometry of the NMDA receptor. The latter possibility seems more feasible since by analogy with other ligand-gated ion channels i.e. \textit{Torpedo} nicotinic acetylcholine receptor, the subunit assembly is not a random process but a highly controlled mechanism (Green and Millar, 1995).

It is not known how many NMDA receptor subunits constitute any native NMDA receptor, though there is compelling evidence as stated above, that they are heteromeric assembles of NR1 and NR2 subunits (Monyer et al., 1994). \textit{In situ} hybridisation and
immunocytochemistry have demonstrated that the expression of the NR1 subunit is ubiquitous in the brain. In contrast, the NR2 subunits are differentially expressed in various regions in the CNS (see Introduction) (Monyer et al., 1994). These results are consistent with the idea of diverse types of NMDA receptor complexes distributed in the CNS. While some native NMDA receptors appear to contain more than one type of NR2 subunit (Luo et al., 1997), there are also conflicting results that suggest that the dominant NMDA receptor complexes in the adult rat cortex are represented by NR1 co-associated with either an NR2A or NR2B subunit (Blahos and Wenthold, 1996; Chazot and Stephenson, 1997a). Furthermore, heterogeneity of the NMDA receptor is further complicated by evidence that suggests that more than one NR1 splice variant can co-assemble within the same complex (Sheng et al., 1994; Blahos and Wenthold, 1996; Chazot and Stephenson, 1997a). However, studies on native receptors have allowed biophysical and pharmacological properties to be equated with particular NMDA receptor subunits. For example, NR1-1a/NR2A and NR1-1a/NR2B heteromeric receptors expressed in *Xenopus* oocytes, exhibited principal conductances of 40 pS and 50 pS. This profile was indistinguishable from hippocampal CA1 pyramidal cells (Stern et al., 1992). The single channel characteristics of the NR1-1a/NR2C channels with conductances of 19 pS and 36 pS, were markedly similar to the native channels of the large cerebellar granule cells. The single channel properties of recombinant NR1-1a/NR2D receptors are very distinct including low-conductances, extended opening times, low sensitivity to Mg$^{2+}$ and offset decay time constants of ~5000 ms (Cull-Candy et al., 1998). These characteristic properties were reciprocated by neonatal purkinje cells. Thus these number of studies form the rationale for
the characterisation of heteromeric NMDA receptor complexes in this thesis, since their pharmacological properties may be representative of native receptors. However, as stated above, reports have shown the existence of NMDA receptors comprising of more than one NR2 subunit co-assembled within the same receptor oligomer (Chazot and Stephenson, 1997a; Luo et al., 1997). However, the relative proportions of native NMDA receptors which contain one type versus two types of NR2 subunits per receptor complex represent a minor subpopulation (Chazot and Stephenson, 1997a).

4.3 SECTION TWO: THE DETERMINATION OF NMDA RECEPTOR-MEDIATED CELL DEATH POST-TRANSFECTION

Prior studies on recombinant NR1-1a/NR2A and NR1-1a/NR2B receptor expression in mammalian cells showed a need for NMDA receptor antagonist protection against cell death (Cik et al., 1993). The observed cytotoxicity is thought to be related to the Ca\(^{2+}\) permeability of the NMDA receptor channel that can lead to excitotoxicity (Choi, 1988). One of the characteristic features of the NMDA receptor is its voltage-dependent Mg\(^{2+}\) block at the resting potential of -80mV. However, the resting potential of HEK 293 cells has been determined to be in the range of -15 to -50 mV (T. Smart personal communication) which should cause a reduced Mg\(^{2+}\) block of the receptor. This observation suggests that under conditions consonant with the high probability of NMDA receptor channel opening i.e. high concentrations of both glycine and glutamate in the cell culture media, the recombinant channel will be highly permeable to Ca\(^{2+}\) ion influx, which could
probably lead to events that culminate in cell cytotoxicity. However, co-expression of both NR1-1a/NR2C and NR1-1a/NR2D receptors did not result in cell cytotoxicity post-transfection although specific [3H] MK801 radioligand binding activity and similar levels of [3H] MDL105,519 binding sites were found to cell homogenates expressing NR1-1a/NR2C and NR1-1a/NR2D receptors from cells transfected in parallel to these cytotoxicity studies. These latter radioligand binding results show that the failure to detect cell cytotoxicity is not due to the lack of expression of both NR1-1a/NR2C and NR1-1a/NR2D receptors. Chazot et al., (1994) also observed that the co-expression of the NR1-1a/NR2C receptor in HEK 293 cells did not result in significant cell death. These contrasting results of cell cytotoxicity post-transfection between NR1-1a/NR2A, NR1-1a/NR2B and NR1-1a/NR2C receptors were postulated to be due to differences in Ca\(^{2+}\) ion permeabilities (Grant et al., 1997). This was demonstrated by measuring intracellular levels of Ca\(^{2+}\) ions in HEK 293 cells co-transfected with various combinations of NMDA receptor clones using the Ca\(^{2+}\)-sensitive bioluminescent protein, aequorin, followed by single cell imaging with the fluorescent calcium indicator, fluoro-3 (Grant et al., 1997). Agonist application to NR1-1a/NR2A or NR1-1a/NR2B transfected cells elicited robust rises in intracellular levels of Ca\(^{2+}\) ions. In contrast, no detectable increases in intracellular Ca\(^{2+}\) responses were observed in NR1-1a/NR2C transfected cells. These results may be explained by a low Ca\(^{2+}\) ion permeability of NR1-1a/NR2C channels compared with both NR1-1a/NR2A and NR1-1a/NR2B receptors (Grant et al., 1997). However, Ca\(^{2+}\) permeability estimated by measuring reversal potentials was comparable among all four NR1-1a/NR2 heteromeric channels (Monyer et al., 1994). A study by
Burnashev et al., (1995) suggests that Ca$^{2+}$ may act as a permeant blocker of both NR1-1a/NR2A and NR1-1a/NR2C channels with the NR1-1a/NR2C channel exhibiting a more prominent block providing an alternative explanation for the cytotoxicity observations. Nonetheless, the latter rationale is at variance when both electrophysiological and radioligand binding results are considered. Both NR1-1a/NR2C and NR1-1a/NR2D receptors have the highest affinity for glycine compared with NR1-1a/NR2A and NR1-1a/NR2B complexes (Kutsuwada et al., 1992; Wafford et al., 1993; Laurie and Seeburg, 1994; Priestley et al., 1995; Buller and Monaghan, 1997). Additionally as mentioned above, NR2D subunit-containing receptors have very distinct single channel properties which include extended opening times and slow deactivation kinetics (Cull-Candy et al., 1998). These correlated results would suggest that there would be higher probability of channel opening of NR1-1a/NR2C, NR1-1a/NR2D channels in comparison with NR1-1a/NR2A and NR1-1a/NR2B receptors. However, electrophysiological studies have shown that both NR1-1a/NR2C and NR1-1a/NR2D receptors have lower conductance states in comparison to NR1-1a/NR2A and NR1-1a/NR2B complexes which may partly explain the differences in cell death (Stern et al., 1992; Cull-Candy et al., 1998).
4.4 SECTION THREE: CHARACTERISATION OF [3H] MDL105,519
RADIOLIGAND BINDING TO NATIVE AND CLONED NMDA RECEPTORS

4.4.1 [3H] MDL105,519 SATURATION RADIOLIGAND BINDING ASSAYS

Previously, radioligand binding properties of [3H] MDL105,519 have been described a single high affinity of $K_D = 3.7 \pm 0.2$ nM for adult rat forebrain membranes with a pharmacological profile that is consistent with that of the glycine site of the NMDA receptor (Baron et al., 1996). In this study, the NMDA receptor subtype-selectivity of [3H] MDL105,519 was studied by characterising its binding properties to cloned NMDA receptors transiently expressed in HEK 293 cells.

4.4.1.1 [3H] MDL105,519 saturation radioligand binding to NR1 subunits expressed in HEK 293 cells

The radioligand binding of [3H] MDL105,519 to NR1-1a and NR1-2a subunits was saturable and of high affinity. The kinetically derived $K_D$ value of $2.5 \pm 0.3$ nM for native forebrain membranes (Chazot et al., 1998), was in agreement with the equilibrium $K_D$s determined from the saturation curves of [3H] MDL105,519 binding to both NR1-1a ($3.2 \pm 1.9$ nM) and NR1-2a ($2.7 \pm 2.0$ nM) splice forms. In contrast, the affinity of glycine for NR1 subunits has been reported to be significantly lower in comparison to its affinity for
native rat cortex/hippocampal P2 membranes (Kᵢ = 580 nM versus Kᵢ = 139 nM respectively) as determined by the competition of [³H] MDL105,519 radioligand binding (Siegel *et al.*, 1996; Chazot *et al.*, 1998). The disparities in affinity between the agonist and antagonist molecules for the NR1 subunit may be accounted for by the structural differences between the ligands. In contrast to glycine, [³H] MDL105,519 incorporates multiple sites for hydrophobic and hydrogen binding interactions, thus it follows that its affinity may be less dramatically altered by, NR1 subunit expression.

Rosenthal transformations of the saturation data showed that the radioligand bound to a single population of receptors. These results are in agreement with previous binding studies where other classes of glycine site antagonists including [³H] 5,7 DCKA and [³H] L689,560 which both bind with a single high affinity to the NR1-1a subunit when expressed alone in mammalian cells (Lynch *et al.*, 1993; Grimwood *et al.*, 1995). Table 4.1 summarises both the results presented here and those from the literature. As stated above, these results further substantiate that the major determinants for the binding of glycine site antagonists resides on the NR1 subunit (Grimwood *et al.*, 1995; Siegel *et al.*, 1996; Chazot *et al.*, 1998). In agreement with radioligand binding studies, mutational analysis also suggested that the major molecular determinants for glycine binding reside on the NR1 subunit (Kuryatov *et al.*, 1994). Several amino acids in the NR1 subunit involved in the putative pharmacophore of glycine have been identified. These amino acids are found in two discontinuous segments of approximately 150 amino acids termed S1 (amino acids 379-526) and S2 (amino acids 645-775) (see Introduction; Kuryatov *et al.*, 1994; Hirai *et al.*, 1996; Uchino *et al.*, 1997; Wood *et al.*, 1997). It would be of interest to investigate whether
other splice forms of the NR1 subunit influence the affinity of [3H] MDL105,519 for the NR1 subunit. The NR1 splice cassettes are not directly located within in the S1 and S2 domains although, they could alter the tertiary folding of the NR1 polypeptide and thus affect the affinity of glycine site ligands for the NR1 subunit. However, results demonstrated that the C1 exon present in the NR1-1a splice variant does not effect [3H] MDL105,519 radioligand binding affinity for the NR1 subunit (Chazot et al., 1998). Furthermore, preliminary findings have also shown that there were no significant differences in affinity of [3H] MDL105,519 radioligand binding to the NR1-4b splice form (which incorporates the N1 exon) in comparison to the NR1-1a subunit (L.M. Hawkins personal communication).

4.4.1.2 [3H] MDL105,519 saturation radioligand binding to heteromeric NR1-1a/NR2 subunit combinations expressed in HEK 293 cells

[3H] MDL105,519 radioligand binding exhibited saturable and high affinity binding to heteromeric NMDA receptor complexes expressed in HEK 293 cells. Rosenthal transformations of the saturation data showed that the radioligand bound to a single population of receptors. Lynch et al., (1993) also reported that [3H] 5,7 DCKA radioligand binds to heteromeric NMDA receptors with a single high affinity. No significant differences were found for the affinity of [3H] MDL105,519 radioligand binding to NMDA receptor splice forms or heteromeric complexes. The characteristics of [3H] MDL105,519 i.e. its similar high affinity for the splice forms and heteromeric NMDA receptor putative
complexes thus make it an ideal radioligand to investigate the possible NMDA receptor subtype-selectivity of glycine site antagonists in competition radioligand binding assays.

4.4.2 \([^3]H\) MDL105,519 COMPETITION RADIOLIGAND BINDING TO BOTH CLONED AND NATIVE NMDA RECEPTORS BY MDL105,519, GV150,526A AND GV196,771A

4.4.2.1 \([^3]H\) MDL105,519 competition radioligand binding to both cloned and native NMDA receptors by MDL105,519, GV150,526A and GV196,771A as analysed by the sigmoidal competition model

All antagonists, MDL105,519, GV150,526A and GV196,771A, displaced \([^3]H\) MDL105,519 radioligand binding with high affinity from NR1-1a, NR1-2a and NR1-1a/NR2 receptors expressed in HEK 293 cells and adult rat forebrain membranes. Overall, GV196,771A had an approximate 10-fold lower affinity than both MDL105,519 and GV150,526A for all NMDA receptor subtypes studied including those expressed in adult rat forebrain. The affinity of MDL105,519 and GV150,526A for NR1-1a/NR2A and NR1-1a/NR2B receptors and native membranes were not significantly different. However, for GV196,771A, the \(K_i\) for adult rat forebrain membranes was intermediate between the \(K_i\) of NR1-1a/NR2A and NR1-1a/NR2B receptors as determined from the sigmoidal fit (Tables 3.10 and 3.11). The affinity determined for GV196,771A for adult rat forebrains reflects the relative levels of expression of NR1/NR2A and NR1/NR2B receptor subtypes.
These findings are in agreement with previous studies where it was shown that GV150,526A and GV196,771A are high affinity ligands for the strychnine-insensitive glycine binding site of the NMDA receptor (Mugnaini et al., 1997; Quartaroli et al., 1999). As with MDL105,519, the C1 exon present in the NR1-1a but not the NR1-2a splice variant, has no effect on the affinity of either GV150,526A or GV196,771A for the NR1 subunit.

4.4.2.2 [³H] MDL105,519 competition radioligand binding to both cloned and native NMDA receptors by GV150,526A and GV196,771A as analysed by the two-site competition model

An interesting finding was in the pharmacological profiles of the two glycine site ligands that for GV150,526A in NR1-1a/NR2 combinations and in native brain membranes, the Hill coefficients of all four displacement curves were significantly < 1 and were best fit by a two-site binding model. For GV196,771A, the situation was complex since all displacement curves, i.e. inhibition binding of [³H] MDL105,519 to NR1-1a, NR1-2a, NR1-1a/NR2 or brain membranes yielded Hill coefficients < 1 and was best fit to a two-site model. In contrast to both GV150,526A and GV196,771A, competition profiles of MDL105,519 binding to single and heteromeric NMDA receptor complexes by MDL105,519 were best to a one-site model.

The high affinity site resolved from the inhibition binding of [³H] MDL105,519 by
GV150,526A to NR1-1a/NR2A (0.7 ± 0.2 nM), NR1-1a/NR2B (0.5 ± 0.1 nM) receptors and rat forebrain membranes (2.6 ± 1.2) determined by the displacement of [³H] MDL105,519 was in agreement to the affinity determined by saturation of [³H] GV150,526A radioligand binding to rat cortical membranes (0.8 ± 0.1 nM) (Mugnaini et al., 2000). However, the saturation analysis of [³H] GV150,526A radioligand binding was described to be best fit to a one-site hyperbola, which contradicts the results presented here. These discrepancies may be explained by the narrow range of [³H] GV150,526A used, i.e. 0.3 - 30 nM with the Kᵢ (low affinity) for NR1-1a/NR2A or NR1-1a/NR2B, the most prevalent receptor subtypes being Kᵢ = 80 nM and the inherent difficulty of using [³H] GV150,526A. The latter results in low signal:noise ratios at high [³H] GV150,526A concentrations thus making deviation from one-site binding difficult to detect (Chopra et al., 2000). Furthermore, the low affinity site may not have been detected in the study by Mugnaini et al., (2000) due its fast dissociation kinetics since, a filtration-based protocol was used to separate the unbound radioligand.

Several other glycine site antagonists with diverse chemical structures were studied in [³H] MDL105,519 displacement assays to determine if under the assay conditions used here, they exhibited similar behaviour to GV150,526A or GV196,771A. Table 4.2 compares the results presented in this thesis with published values. Of the five compounds, only L689,560 yielded a one-site fit to NR1-1a and a two-site fit to NR1-1a/NR2A receptors. Grimwood et al. (1992) also reported a Hill coefficient of 1.00 for the displacement of [³H] L689,560 radioligand binding to both NR1-1a and NR1-4a receptors by unlabelled L689,560. No binding information has been published for NR1/NR2 combinations.
However, saturation radioligand binding of $[^3]H$ L689,560 to adult rat membranes yielded a Hill coefficient that was significantly greater than unity. Additionally the rates of dissociation/association were both better fit by a double exponential curve, suggestive of heterogeneity of sites (Grimwood et al., 1992).

In another study, Parsons et al. (1997) found that L689,560 displaced the radioligand binding of $[^3]H$ MDL105,519 to rat cerebral cortical membranes with a Hill coefficient = 1.13. The conditions of the radioligand binding assay differed from those used here, i.e. the buffer was 50 mM Tris-HCl, pH = 8.0 compared to pH = 7.4 and the assay incubations were carried out for 45 min at 4°C compared with 90 min at 4°C used here. Displacement of $[^3]H$ MDL105,519 radioligand binding to rat cerebral cortical membranes by L689,560 should be performed under the conditions employed by Parsons et al., (1997) in order to investigate if the discrepancies in the competition profiles of L689,560 are dependent on the conditions used in the radioligand binding assays.

4.5 SECTION FOUR: INVESTIGATION OF THE BIPHASIC COMPETITION CURVES OBTAINED FROM DISPLACEMENT OF $[^3]H$ MDL105,519 RADIOLIGAND BINDING TO NMDA HETEROMERIC RECEPTORS BY GV150,526A AND GV196,771A

Biphasic, concave inhibition curves of both GV150,526A and GV196,771A to all four NR1-1a/NR2 receptor combinations expressed in HEK 293 cells may be the result of
several different phenomena. For example, impurity and/or stability of the displacing ligand. However, for GV150,526A, the biphasic binding curve is coincident with the presence of the NR2 subunit since displacement assays carried out in parallel and under identical conditions to NR1-1a and NR1-1a/NR2 yielded one-site and two-site fits respectively. In addition, FAB mass spectroscopy showed that both GV150,526A and GV196,771A were stable before and following the radioligand binding assay and that both these ligands were > 99% pure (Figures 3.27-3.28). Thus these correlated findings eliminate ligand impurity and stability as the possible explanation for heterogeneity.

Alternatively, binding to a heterogeneous population of receptors which have different affinities for the ligand; the existence of two binding sites within a receptor with different affinities for the ligand; different states of an apparently single binding site and negative co-operativity may yield shallow displacement curves. Many strategies were used to investigate the delineation of the biphasic nature of the competition curves. The rationale behind these approaches was based on previous studies that have shown that when HEK 293 cells are co-transfected with pCISNR1-1a and pCISNR2A, the expressed receptors are heterogeneous comprising of both unassembled NR1-1a and assembled NR1-1a/NR2A complexes (Chazot and Stephenson, 1997b). The biphasic displacement curves may be explained by both GV150,526A and GV196,771A distinguishing between the two NMDA receptor subpopulations, i.e. NR1-1a and NR1-1a/NR2A thus resulting in biphasic displacement curves. It has been reported that when the NR1-1a subunit is expressed alone, it does not reach the cell surface but it is retained in intracellular occlusions associated with the endoplasmic reticulum (McIlhinney et al., 1998). The techniques used to investigate the
biphasic competition curves of both GV150,526A and GV196,771A, targeted heteromeric NR1-1a/NR2 complexes from unassembled NR1-1a subunit pools.

4.5.1 $[^{3}H]$ MDL105,519 radioligand binding to cell surface NMDA receptors expressed in HEK 293 cells

One such technique used was intact cell radioligand binding to study the displacement by GV150,526A and GV196,771A of $[^{3}H]$ MDL105,519 binding to cell surface receptors rather than to homogenates of transfected cells where putative intracellular pools would be accessed by a hydrophilic radioligand. There are many criteria that need to be fulfilled in order to perform cell surface radioligand binding assays. Firstly, transfected cells must remain attached to the culture dishes throughout the radioligand binding assay. This latter consideration was ensured by pre-coating the petri dishes with a positively charged polymer, poly-L-lysine, which interacts with the negatively charged scalaric acid moieties on the membranes of HEK 293 cells. Secondly, an isotonic Lockes buffer solution was used in the intact cell radioligand binding assay to maintain any ionic or pH gradient that exists across the plasma membrane and to maintain the integrity of the cell. Moreover, the choice of radioligand was a critical factor in cell surface radioligand binding assays. $[^{3}H]$ MDL105,519 was employed for many reasons including its high affinity for the glycine binding site thus increasing the signal:noise ratio and consequently permitting the use of a low concentration of radioligand in the assay to reduce non-specific radioligand binding. Furthermore, the distribution coefficient of $[^{3}H]$ MDL105,519 in an octanol:buffer
system showed that the radioligand was a hydrophilic compound and thus would not be expected to transverse the plasma bilayer at 4°C (Table 3.15). Finally, since [³H] MDL105,519 is an antagonist, any changes to the receptor in the radioligand binding assay including desensitisation and down-regulation would be minimised.

Additional conditions which were employed in the preparation of intact cells for cell surface radioligand binding assays included, trypsin-treatment of transfected cells 1 h post-transfection. Cells were subjected to a trypsin-treatment post-transfection to pool cells from different flasks to minimise any differences in the transfection efficiencies also to permit cells to be plated at an equal density on culture dishes.

Following the determination of the optimal conditions to be employed in intact cell surface radioligand binding assays, the level of [³H] MDL105,519 radioligand bound to both NR1 and NR1-1a/NR2A receptors prepared as either cell homogenates or intact cells were compared. The level of specific [³H] MDL105,519 radioligand bound to both NR1-1a and NR1-1a/NR2A receptors was approximately 2-3-fold higher for membranes prepared as cell homogenates compared to intact cells (Figure 3.37). This latter finding shows that the radioligand bound to intact cells would only have access to bind to cell surface receptors whereas in cell homogenates the radioligand could label both the cell surface NR1-1a/NR2A receptors and intracellular pools of NR1-1a and NR1-1a/NR2A receptors.

Interestingly, a similar level of specific [³H] MDL105,519 radioligand bound was found to intact cells expressing NR1-1a alone or in combination with the NR2A subunit. This was a very unexpected finding, since as stated above, it has been reported that when the NR1-1a subunit is expressed alone, it is retained in intracellular occlusions (McIlhinney et al.,
1998). However, the study of Ehlers et al. (1995) revealed that NR1 splice forms containing the first COOH-terminal cassette, NR1-1a and NR1-2a, when expressed alone in fibroblast cells were located in discrete, receptor-rich domains associated with the plasma membrane in contrast to the splice variants lacking this cassette (NR1-3a and NR1-4a) which were distributed throughout the cell. This is in direct conflict with McIlhinney et al. (1998) but Ehlers et al. (1995) state that the resolution of their studies does not permit them to distinguish between NR1-1a being expressed on the cell surface or NR1-1a being localised at the intracellular face of the membrane.

However, the experiments described have ascertained that \[^{3}H\] MDL105,519 radioligand binding occurs to cell surface NR1-1a rather than to intracellular NR1-1a subunit pools in intact cells expressing NR-1a alone. For example, trypsin dissociation post-transfection does not effect the cell viability of intact HEK 293 cells expressing NR1-1a or NR1-1a/NR2A receptors (Figure 3.38). These findings support the fact that trypsin-treatment of cells post-transfection does not damage the plasma membrane and thereby will not allow access of \[^{3}H\] MDL105,519 to intracellular NR1-1a subunits.

In addition, no significant endogenous uptake of \[^{3}H\] MDL105,519 in untransfected intact cells was detected (Figure 3.41). Analogously, there was no inhibition of any uptake of glycine by transporter systems in the membranes of intact HEK 293 cells expressing the NR1-1a subunits using L-alanine as demonstrated by \[^{3}H\] MDL105,519 radioligand binding (Saier et al., 1988) (Figure 3.39). These results provide additional support that \[^{3}H\] MDL105,519 radioligand binding occurs to cell surface NR1-1a rather than to intracellular NR1-1a subunit pools. Alternatively, it may be argued that the concentration of glycine (1
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mM) used in the latter experiments may have been at saturating level. Therefore, L-alanine would be unable to inhibit any glycine uptake and thus no difference would have been observed in the levels of specific [³H] MDL105,519 radioligand binding to intact cells expressing the NR1-1a subunit in the absence or presence of L-alanine. Future studies could investigate whether any intracellular glycine uptake can be minimised by L-alanine at glycine concentrations lower than that used in the present study (1mM). However, the distribution coefficient of [³H] glycine in an octanol:buffer system showed that the radioligand was a hydrophilic compound and thus would not be expected to transverse the plasma bilayer (Table 3.15). Hence this reinforces that specific [³H] MDL105,519 radioligand binding to intact cells expressing the NR1-1a subunit is likely to occur at the cell surface.

Conclusive evidence is required to determine whether the NR1-1a subunit is trafficked to the plasma membrane when expressed alone in HEK 293 cells. Immunocytochemistry could be used to address this point. HEK 293 cells transfected with the NR1-1a clone could be immunostained with antibodies against putative extracellular and intracellular NR1 domains under fixation conditions revealing either surface or both surface and intracellular labelling. Alternatively, the distribution of green fluorescent protein (GFP)-tagged NR1 subunits could be used to investigate the subcellular distribution of the NR1 subunit post-transfection in mammalian cells. HEK 293 cells could be transfected with a plasmid expressing fusion proteins of the GFP attached to the intracellular C-terminal of the NR1 subunit. The image of the cellular location of the NR1 subunit, 24 h post-transfection could then be monitored by autofluorescence.
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In summary, both the intact cell surface radioligand binding and control experiments indicate that the NR1-1a subunit may be expressed at the cell surface. Thus the cell surface receptors expressed following the co-transfection in HEK 293 cells with pCISNR1-1a/pCISNR2A may comprise of a heterogeneous population of receptors i.e., NR1-1a, and NR1-1a/NR2A complexes. Thus clearly these findings invalidate the rationale to use the technique of intact cell radioligand binding to investigate the displacement by both GV150,526A and GV196,771A of $[^3]$H MDL105,519 radioligand binding to cell surface receptors.

4.5.2 Allosteric modulation by L-glutamate of the binding of GV150,526A and GV196,771A to NR1-1a/NR2A receptors expressed in HEK 293 cells and native forebrain membranes determined from the competition radioligand binding of $[^3]$H MDL105,519

The inhibition binding of $[^3]$H MDL105,519 by both GV150,526A and GV196,771A to heteromeric NR1-1a/NR2 receptors resulted in biphasic displacement curves. This may be due to the possibility that GV150,526A and GV196,771A can distinguish between the two NMDA receptor subpopulations, i.e. NR1-1a and NR1-1a/NR2A. To address the one versus two-site binding models, L-glutamate was used to modulate allosterically the affinity of GV150,526A and GV196,771A for assembled NR1-1a/NR2A receptors. L-glutamate interacts with assembled NR1-1a/NR2A receptors but virtually lacks affinity for unassembled NR1 subunits (Laurie and Seeburg, 1994) as the L-glutamate binding site has
been localised to the NR2 subunit (Laube et al., 1997). An ideal characteristic of [³H] MDL105,519 is that its affinity for NR1-1a subunits or to native membranes is unaltered by L-glutamate (Baron et al., 1996; Siegel et al., 1996). Consequently, [³H] MDL105,519 was employed to investigate any allosteric influences of L-glutamate on the affinities of both GV150,526A and GV196,771A for both heteromeric NMDA and native receptors. No significant differences were found between the competition curves of inhibition binding of [³H] MDL105,519 to NR1-1a/NR2A receptors by both GV150,526A and GV196,771A ± 10-1000 µM L-glutamate under equilibrium conditions (Figure 3.31a). These results may be reconciled with the conditions used in the assay. For example, at equilibrium any allosteric modulation by L-glutamate on the affinities of both GV150,526A and GV196,771A could be masked as parallel changes in both the association and dissociation rate constants of the ligands could occur in the same direction. The experiments were then repeated under non-equilibrium conditions using adult rat forebrain membranes. Radioligand binding studies have shown that [³H] L-glutamate exhibits a higher affinity for NR1/NR2B receptors compared to NR1/NR2A receptors, these two receptors being the prevalent subtypes expressed in adult rat forebrain (50 nM versus 100 nM respectively) (Laurie and Seeburg, 1994). However, the concentration of L-glutamate employed for the allosteric experiments was of the correct order of magnitude i.e 10-1000 µM L-glutamate, to effect both NR1-1a/NR2A and NR1-1a/NR2B receptor subtypes. The presence of L-glutamate under non-equilibrium conditions resulted in the inhibition binding of [³H] MDL105,519 to adult rat forebrain membranes by GV150,526A to be best fit to a one-site model compared to a two-site fit (Figure 3.31b). In contrast, in the absence of L-glutamate
the displacement binding of GV150,526A was best fit to a two-site compared to a one-site model (Figure 3.31b). In the presence of 100 μM L-glutamate, the $K_i$ determined for GV150,526A was 8-fold higher than the $K_i$ calculated in the absence of L-glutamate (Table 3.14).

These results suggest, firstly, that L-glutamate allosterically decreased the affinity of GV150,526A for heteromeric NMDA receptor subtypes. The results are consistent with previous findings that have shown that L-glutamate allosterically decreases the binding in rat brain membranes of some glycine site antagonists (Grimwood et al., 1992; 1993). Secondly, since L-glutamate does not modulate [³H]MDL105,519 radioligand binding to native membranes (Baron et al., 1996) the radioligand would label all NMDA receptor complexes in the membrane preparation i.e unassembled NR1 and assembled NR1/NR2A and NR1/NR2B receptors. Therefore GV150,526A must still bind to all the heterogenous populations of NMDA receptors as the competition profiles of GV150,526A + L-glutamate resulted in 100% inhibition of [³H]MDL105,519 radioligand binding.

A simple explanation to account for the biphasic competition curves obtained in the absence of glutamate would be that GV150,526A has lower affinity for NR1/NR2 receptors compared to unassembled NR1 subunits. This would also assume that these two types of receptors would be expressed at approximately equimolar concentrations. The high affinity $K_i$ values for the two-site fit are in approximate agreement with the $K_i$ values for binding to NR1 single subunits (cf. Tables 3.6 and 3.9)

However, the analysis of the competition curve of GV150,526A binding to rat forebrain membranes in the presence of L-glutamate shows that the situation is complex and cannot
be accounted by the latter hypothesis. In the presence of 100 μM L-glutamate, the $K_i$ for GV150,526A was 8-fold higher than the $K_i$ determined in the absence of L-glutamate (Table 3.14). Therefore L-glutamate could have allosterically decreased the affinity for GV150,526A binding to the heteromeric complexes, such that the affinity difference of GV150,526A for both heteromeric and single NR1 subunits may be too small to be resolved by the displacement assay thus resulting in a monophasic competition curve. Accordingly this would suggest that assembled NR1/NR2 receptors do not exclusively represent one of the two sites resolved by the inhibition binding of $[^3H]$ MDL105,519 to native receptors by GV150,526A. Hence, the sites identified in the biphasic competition curves are due to interacting receptor complexes or co-operativity in an single receptor oligomer and not independent heterogenous binding sites i.e. unassembled NR1 versus assembled NR1/NR2 receptors constituting either of the two sites resolved, as previously suggested.

To resolve this issue of whether these sites are interacting by a co-operative manner or independent of each other further kinetic experiments could be performed. The rate of dissociation of $[^3H]$ GV150,526A binding to NR1-1a/NR2A receptors expressed in HEK 293 cells could be compared under two conditions, infinite dilution of the dissociation of the radioligand versus infinite dilution in the presence of an excess concentration of unlabelled GV150,526A. A centrifugation assay should be employed to separate the unbound radioligand in order to detect the low affinity site labelled by $[^3H]$ GV150,626A. The results from both the dissociation reactions are graphically represented by plotting $\log_{10}$ of the concentration of bound ligand /concentration of bound ligand at equilibrium at time,
t = 0 against time. Dissociation of $[^3]$H GV150,626A initiated by infinite dilution should produce a curvilinear plot. Results from the dissociation of $[^3]$H GV150,626A initiated by infinite dilution in the presence of an excess concentration of unlabelled GV150,526A, could either yield either a linear or curvilinear plot, depending on the interaction of ligand with the receptor. A resultant linear plot would be suggestive of negative co-operative since the radioligand binding of $[^3]$H GV150,526A to NR1-1a/NR2A receptors will decrease the overall affinity of the receptor population and accelerate the rate of radioligand dissociation. This is because a receptor population that exhibits cooperative behaviour nonetheless is characterised by a single $K_D$ value at saturating receptor occupancy. In contrast, a curvilinear dissociation plot indicates the existence of an independent population of receptors that bind to the radioligand with different dissociation rate constants.

Future studies should also be performed to determine whether cell homogenates from cells transfected with NR1-1a and NR2A clones contain assembled and unassembled NR1-1a complexes in approximately equal proportions since the two sites resolved from the inhibition binding of $[^3]$H MDL105,519 by GV150,526A and GV196,771A, were present in approximately equal percentages (Tables 3.9 and 3.10). Detergent extracted receptors i.e. NR1-1a and NR1-1a/NR2A, from HEK 293 cells transiently co-transfected with NR1-1a and NR2A clones, could be subjected to an anti-NR2A immunoaffinity purification. The elution from the latter purification can be quantified for the percentage unassembled NR1-1a subunits, with respect to unbound and bound NR1-1a subunits (total NR1-1a immunoreactivity), by immunoblotting using the respective anti-NR1-1a subunit-specific antibody.
4.6 SECTION FIVE: COMPARISON OF THE CHEMICAL STRUCTURES OF MDL105,519, GV150,526A, GV196,771A AND L689,560

As above, the displacement of $[^3]$H MDL105,519 radioligand binding to all heteromeric receptors by MDL105,519 was significantly best fitted to a one-site model. In contrast, the competition profiles of GV150,526A, GV196,771A and L689,560 binding to heteromeric complexes was better fitted to a two-site compared to a one-site model. Physiological and molecular biological studies are both consistent with there being at least two high affinity glycine binding sites per NMDA receptor oligomer. Consequently the inhibition binding of $[^3]$H MDL105,519 by GV150,526A, GV196,771A and L689,560 to NR1-1a/NR2 receptors may exert a steric influence to the binding of the second GV150,526A, GV196,771A or L689,560 molecule resulting in negative co-operativity. This could be a consequence of the disparate structural features between these ligands. Detailed analysis of the chemical structures of MDL105,519 with both GV150,526A and GV196,771A revealed distinct structural differences between the ligands as highlighted in Figure 4.1. For example, both GV150,526A and GV196,771A exist as trans stereo-isomers with phenylamide moieties conjugated to the phenyl ring. The trans isomer configuration contributes to a greater conformational entropy to these compounds since the lone pair of electrons present on the N atom of the amide group interacts with the $\pi$-electron system of phenyl ring. In addition, both compounds have six spacer carbon atoms separating the indole group from the phenyl ring. In contrast, MDL105,519 is a cis stereoisomer with a four spacer carbon atoms between the indole nucleus and the phenyl group.
One structural similarity shared by GV150,526A, GV196,771A and L689,560, that is not present in the other four ligands characterised in the above section i.e., glycine, 7-CLKA, 5,7-DCKA, L701,324, is that these ligands all have phenylamide groups. Figure 4.2 highlights the parts of the molecules shared by all the antagonists investigated. Conceivably, the lone pairs of electrons on N atom of the amide group may ionically interact with the positive group present on the NMDA receptor and consequently may contribute to the distinct inhibition profiles of these ligands.

Previously, it has been shown that the displacement of \([\text{^3}H]\) MDL105,519 radioligand binding by GV196,771A to NR1-1a subunits was best fit by a two-site model. Whereas the inhibition binding of \([\text{^3}H]\) MDL105,519 to NR1-1a subunits by both GV150,526A and L689,560 resulted in a competition curve best fit to a one-site binding model. Comparison of the chemical structures of L689,560, GV150,526A and GV196,771A show that the former compounds contain a secondary amide groups. In contrast, GV196,771A has a tertiary amide indole group substituted on the phenyl ring. These structural differences between GV150,526A, L689,560 and GV196,771A may provide an insight to the distinct competition profiles of these ligands binding to single NR1 subunits. The bulky indole group of GV196,771A may sterically hinder the binding of the second GV196,771A molecule to (NR1-1a)_n receptors resulting in negative co-operativity and a biphasic displacement curve.

It would be of interest to determine if complexes of NR1-1a subunit exist from cells transfected with NR1-1a clones. Gel filtration or sucrose density gradient sedimentation could be used to separate different complexes of the solubilised NR1-1a receptor subunits.
The resulting fractions could be analysed by immunoblotting using anti-NR1-1a subunit-specific antibodies.

4.7 SECTION SIX: GV150,526A AND GV196,771A NMDA RECEPTOR SUBTYPE SELECTIVITY WITH REGARD TO THEIR THERAPEUTIC PROFILES

Interestingly, the presence of the NR2 subunit does not affect the affinity of $[^3H]$MDL 105,519 for the glycine recognition site on the NR1 subunit (section 4.4.1.2) whereas glycine binding to heteromeric NMDA complexes has been shown to exhibit marked discrimination between subunit assemblies. For example, radioligand binding assays show that glycine has ~2-3 fold higher affinity for NR1/NR2C receptors compared with NR1/NR2A and NR1/NR2B complexes as demonstrated by inhibition of $[^3H]$5,7 DCKA radioligand binding (Laurie and Seeburg, 1994). These results suggest that presumably the bulky antagonist molecule may negate any steric influences of the NR2 subunit, whereas for a smaller molecule such as glycine the structural effects of the NR2 subunit are more pronounced.

However, there is some evidence which suggests that NR2 subunits may effect the affinity of glycine site antagonist binding to heteromeric NR1/NR2 complexes. For example, functional studies have reported that L689,560 has a five-fold selectivity for human NR1-1a/NR2A compared to human NR1-1a/NR2B receptors in inhibiting NMDA and D-serine-induced responses in *Xenopus* oocytes (Hess et al., 1996). More recently, it was shown that
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$[^{3}H]$ CGP61594, a glycine site antagonist which is also a photoaffinity ligand, preferentially labelled NR2B-subunit containing NMDA receptors although the incorporated radioactivity was associated with only the $M_r \sim 120 \,000$ NR1 subunit (Honer et al., 1998). A low affinity reversible binding site for $[^{3}H]$ CGP61594 was found in rat cerebellar membranes. It was attributed to binding to NR1/NR2A, NR1/NR2C or both subtypes of receptor since these are the subunits expressed in adult cerebellum (Honer et al., 1998).

Furthermore, competition of $[^{3}H]$ MDL105,519 radioligand binding to heteromeric NR1-la/NR2 receptors by both GV150,526A and GV196,771A showed a small but significant NMDA receptor subtype-selectivity (section 4.4.2.1). When the $K_i$ values for GV150,526A for the sigmoidal binding model were compared between NR1-1a and NR1-1a/NR2 combinations, the presence of the NR2 subunit did not significantly effect antagonist affinity except for NR2C in NR1-1a/NR2C receptors, which had a slightly lower affinity for GV150,526A. In contrast to GV150,526A, GV196,771A exhibited 3-4-fold lower affinity for NR1-1a/NR2A receptors compared to the other NR1-1a/NR2 complexes. When the curves were analysed by the better fit two-site model, this selectivity was due to a reduced affinity of GV150,526A for the NR1-1a/NR2C high affinity site (Table 3.9). The low affinity of GV196,771A for NR1-1a/NR2A receptors was attributable to the low affinity binding component. However, analysis of the curves would suggest that the overall reduced affinity was due to both the high and low sites (Table 3.10).

The NMDA receptor subtype-selectivity determined for GV150,526A and GV196,771A by electrophysiological studies to cloned heteromeric NR1-1a/NR2 receptors expressed in HEK 293 cells, is in direct contrast to the results presented here (C. Carignani personal
communication) in that the rank order of increasing potency for GV150,526A was NR1-1a/NR2D > NR1-1a/NR2B = NR1-1a/NR2C > NR1-1a/NR2A and for GV196,771A was NR1-1a/NR2D > NR1-1a/NR2C > NR1-1a/NR2B > NR1-1a/NR2A. The discrepancies in these findings may be a consequence of the different methodologies used to generate these results. For example, electrophysiological experiments are performed in the presence of NMDA which may mediate an allosteric effect on the affinity of glycine site antagonists. Alternatively, mechanical disruption to the membrane during patch formation has been shown to lead to oxidation of the NMDA receptor (Brimecombe et al., 1997). Thus, between radioligand binding and electrophysiological assays, different oxidation states of the NMDA receptor may have been studied which may have differing affinities for both GV150,526A and GV196,771A. Furthermore, electrophysiological experiments performed at a physiological temperature, in contrast the radioligand binding studies carried out here were at 4°C. Hence, different kinetic states of the receptor may influence the affinity of glycine site antagonists for the NMDA receptor. Finally in the study by C. Carignani, rat NR2 cDNAs were rather than mouse NR2 cDNAs as used here. As stated above, the predicted amino acid sequences for the mature polypeptides of the rat NR2 subunits share 88% amino acid sequence identity with their mouse counterparts. The disparate amino acids between the rat and mouse subunits could either have a potential steric effect or modulate the hydrogen bond network on the NR1 subunit and accordingly, effect the antagonist affinity.

However, the moderate selectivity exhibited by GV196,771A presented in this thesis is in accordance with an electrophysiological study by Quartaroli et al., (1999) where it was
demonstrated that the rank order of potency of GV196,771A in the adult rat brain was spinal cord > hippocampus > cortex. The potency of GV196,771A in the different areas of the rat brain may be attributed to the distinct pattern of expression of the NR2A subunit. Immunohistochemistry experiments have shown that NR2A-containing receptors are abundantly expressed in the cortex and hippocampus but are sparse in the spinal cord (Wenzel et al., 1995; P.L. Chazot personal communication).

This moderate NMDA receptor subunit-selectivity exhibited by both GV150,526A and GV196,771A may have beneficial therapeutic implications. For example, GV150,526A, progressed to Phase III clinical trials for treatment of stroke. This compound showed a reduction in the infarct area and protection against somatosensory-evoked potentials in the MCAo model of focal ischemia in the rat (Bordi et al., 1997). Furthermore, it is devoid of amnestic side effects at doses well above the neuroprotective range of action (Bordi et al., 1996). The two-four lower affinity of GV150,526A for NR1-1a/NR2C receptors in comparison to other heteromeric NMDA receptor complexes investigated may aid the treatment for neurodegenerative disorders. This is because channels comprising of NR1-1a/NR2C receptors show distinct single channel properties including, higher percentage of time in subconductance states, low main conductance levels and opening times (Cull-Candy et al., 1998). These correlated properties of NR1-1a/NR2C receptors suggest that there would be a low Ca\(^{2+}\) ionic flux associated with open channel. Furthermore, the expression of recombinant NR1-1a/NR2C channels is not cytotoxic which is in direct contrast to NR1-1a/NR2A and NR1-1a/NR2B receptors (section 4.3). Thus under ischaemic conditions, NR1-1a/NR2C channels are unlikely to mediate Ca\(^{2+}\) overload which is associated with
excitotoxicity. Hence, compounds with lower affinity for NR1-1a/NR2C will have reduced propensity for side effects since the physiological role of these receptors may not be blocked. In addition, it has been noted that *in vivo* the granule cell layer of the cerebellum and interneurons in the hippocampal regions of the adult brain that predominately express the NR2C subunit, are brain areas that are more resistant to ischaemic insult (Small *et al.*, 1997).

Studies should be performed to investigate whether GV150,526A exhibits a low affinity for the cerebellum analogous to its affinity determined for cloned NR1-1a/NR2C receptors. Displacement of $[^\text{H}]$ MDL105,519 radioligand binding to cerebellar membranes by GV150,526A, may reveal more than one binding site, as this region contains a heterogenous population of NMDA receptors. The lower affinity site produced by the displacement of $[^\text{H}]$ MDL105,519 by GV150,526A may have an affinity similar to that found for recombinant NR1-1a/NR2C receptors.

In an ischaemic environment, alterations in phosphorylation, redox state, allosteric effects of polyamines, variations in glycine concentrations and proton concentration may selectively modify certain NMDA receptor subunits for which GV150,526A may have increased affinity. It would be of interest to determine any NMDA receptor subtype-selectivity of GV150,526A in ischaemic tissue, by correlating *in situ* hybridisation of NMDA receptor mRNA, using specific oligonucleotide probes with autoradiography of $[^\text{H}]$ GV150,526A.

GV196,771A's *in vivo* properties include a reduction in the pain sensitivity in the second phase of inflammation in the formalin test and it blocks the sensitisation caused after
chronic constriction injury of the left sciatic nerve (CCI) in rat. Moreover, GV196,771A did not induce learning and motor deficits (Quartaroli et al., 1999). This ligand progressed to clinical Phase IIb for the treatment of hyperalgesia.

One possible explanation for the reduced side effect profile of GV196,771A as mentioned above could be attributed to its is 3-4-fold lower affinity for NR1-1a/NR2A receptors in comparison to other heteromeric complexes investigated. For example, compounds exhibiting lower affinity for NR2A compared with NR2B-containing receptors may suggest a reduced propensity for causing ataxia since, receptors comprising the NR2B subunit are not expressed in the cerebellum (Watanabe et al., 1993; Wenzel et al., 1995). Furthermore, under pathological conditions NR1/NR2B receptors are selectivity potentiated by the excessive release of polyamines following stimulation of dorsal horn neurons by noxious stimuli (Williams, 1997). Thus GV196,771A may selectivity block NR2B-containing receptors without effecting the physiological role of receptors comprising of NR2A-receptor subtypes. Hence, compounds displaying selectivity for NR2B-containing over NR2A-containing receptor subtypes will have a reduced side effect profile (Boyce et al., 1999).

The affinity of GV196,771A for neonatal rat forebrains, which predominately express NR1 and NR2B subunits, could be compared to adult forebrains which comprise NR1, NR2A and NR2B subtypes by the displacement of $[^3H]$ MDL105,519 radioligand binding in future studies (Watanabe et al., 1992; Monyer et al., 1994; Wenzel et al., 1995). In line with the affinity of GV196,771A determined for heteromeric NR1-1a/NR2A and NR1-1a/NR2B complexes a lower affinity for adult membranes would be expected in comparison to
neonatal membranes.

4.8 SECTION SEVEN: FUTURE DIRECTIONS

GV150,526A was the only glycine site antagonist developed to date to progress to clinical Phase III trials for the treatment of stroke. However, these results have been disappointing. These studies show that this compound does not exhibit first-order pharmacokinetics since it binds to the plasma binding protein, bilirubin. Subsequently, GV150,526A has been withdrawn from clinical trials. GV150,526A can serve as a template in the development of the next generation of indole-2-carboxylates-derived glycine site antagonists. A fine balance between the lipophilic nature and the ability to penetrate the blood brain barrier must be reached, in the design of novel compounds superceding GV150,526A. This will ensure that the progression of these compounds is not analogous to the demise of GV150,526A.

As our knowledge of the different physiological roles of NMDA receptor subunits is becoming apparent, non-selective antagonism of NMDA receptors will obviously produce a narrow threshold between neuroprotective effects and unwanted side effects. The therapeutic index of NMDA receptor antagonists could be markedly improved by using subtype-selective ligands. As stated above, for example, NR2B-selective agents, since this subunit is predominately expressed in the hindbrain relative to the forebrain. Therefore, by selectively blocking NR2B-containing NMDA receptors, a reduced propensity for causing motor side effects may be expected. NMDA receptor subtype-selectivity however, is not
restricted to the NR2 subunit. Future studies should also focus in developing NR1 subunit splice variant selective antagonists. Of particular interest in respect to the potential subunit selectivity of glycine site antagonists is the N1 exon, which is located in the extracellular N-terminus of the NR1 subunit.

Optimism for the treatment of neurodegenerative disorders is markedly moving away from the development of glycine site antagonists but towards agents that display state-dependent block of the NMDA receptor. For example, ifenprodil has a higher affinity for the agonist-bound and desensitised states of the NMDA receptor compared to the resting and agonist-unbound states (Mott et al., 1998). This mechanism of blockade could be responsible for the favourable profile of ifenprodil in ischaemic conditions. Furthermore, NMDA antagonists are being development that display an increased potency at low pH. During an ischaemic insult the interstitial pH within the CNS has shown to fall by 0.2 to 1.0 pH units (Traynelis et al., 1995). Hence, these compounds will inhibit the NMDA receptor under conditions of ischaemic damage to a greater extent than at physiological pH. A phenylethanolamine non-competitive NMDA receptor antagonist displaying these properties is exemplified by, (1S,2S)-1-(4-Hydroxyphenyl)-2-(4-phenylpiperidino)-1-propanol (CP101,606) (Mott et al., 1998).

In summary, the competition profiles and NMDA-receptor subtype-selectivity of three glycine site antagonists were characterised using radioligand binding studies in this thesis. Moderate NMDA receptor-subtype selectivity was observed for both GV150,526A and GV196,771A for cloned heteromeric NMDA receptor subtypes. However, the determined selectivity is too small for determination of pharmacological characteristics in vivo. On the
basis of these results presented in this thesis it is proposed that glycine site antagonists may be subdivided into two pharmacological classes, i.e. those that show simple competitive behaviour with regard to inhibition of [3H] MDL105,519 radioligand binding and those that result in complex inhibition curves (Figure 4.3). The two classes of antagonists, could serve as tools in the characterisation of the glycine antagonist binding site on the NMDA receptor. To investigate whether their distinct competition profiles are due to the two types of antagonists binding to separate or overlapping glycine binding site domains on the NR1 subunit, this could be addressed by future studies, by determining whether there is any correlation between the pharmacological profiles of the two subclasses of glycine site antagonists identified. Nonetheless, more compounds will need to be screened to address structure/function relationships in the context of glycine antagonist competition profiles. Alternatively, computational modelling techniques using the X-ray coordinates of PBPs as templates, which share similar 3D structures with ionotropic glutamate receptor subunits (see Introduction) in combination with the specific amino acids identified to be crucial for glycine binding on the NR1 subunit by in vitro mutagenesis, may allow a theoretical 3D model of the NR1 subunit to be constructed. This model may predict how the two subclasses of glycine site antagonists identified here are accommodated in the ligand-binding pocket by different amino acids and thus whether they interact at distinct or overlapping sites. However, these theoretical predicted computational 3D models have a degree of uncertainty associated with them. The results presented in this thesis show that the NR2 subunit influences the competition profiles of some glycine site antagonists for example, inhibition binding of [3H] MDL105,519 by GV150,526A and L689,560 to NR1-
A subunit was shown to be best to a one-site competition model in contrast, NR1-1a/NR2A receptors to a two-site model. Hence, these models need to take into consideration of the allostERIC effect of the NR2 subunit on the glycine binding site of the NR1 subunit to resolve how intra-subunit conformational changes effects the quaternary structure of the glycine binding site. In addition, these theoretical models are projected using site-specific mutations that alter ligand binding affinities. However, these mutated residues may not be directly interacting with the ligand but instead may be effecting the network of interactions between amino acids in the ligand binding pocket. Furthermore, the hypothetical ligand binding pocket of the NR1 subunit would be modelled using the X-ray co-ordinates of PBPs however, these proteins are liganded by glutamate whereas the NR1 subunit contains the structural determinants for glycine binding. Discrete amino acids residues on the NR1 subunit interacting with glycine may be not be considered important when modelling the ligand binding pocket of the NR1 subunit on 3D structure of PBPs.

Nonetheless, the 3D structure of the soluble S1-S2 fragment of the GluR2 subunit bound to kainate solved by X-ray crystallography, was found to be similar to the crystalline structure of various PBPs (Armstrong et al., 1998). This high-resolution structure of the glutamate receptor subunit allowed the identification of residues that mediate agonist specificity and channel dynamics. Analogously, the soluble protein comprising of the S1 and S2 segments of the NR1 subunit has been expressed in a baculovirus system with a pharmacological profile indistinguishable from that of wild-type NR1 subunits expressed in mammalian cells (Ivanovic et al., 1998). Homologous S1 and S2 domains on the NR2 subunit have also been identified that are required for high affinity glutamate binding.
Forthcoming studies could aim to resolve the crystalline structure of heteromeric NR1/NR2 receptors by co-expressing the cDNAs encoding S1 and S2 segments of both NR1 and NR2 subunits in a baculovirus system to obtain large quantities of the functional dimeric ligand binding domain of an NMDA receptor. Future approaches may turn to using these X-ray crystallographic structures as templates for the rationale of drug design, but it remains unclear whether the crystalline structure would represent a desensitised or active channel conformation, since these distinct conformations may dictate different predicted ligand structural profiles. However, conformational changes between the two states may be addressed by obtaining high resolution 3D structures of liganded and unliganded intact receptor oligomers (Pass et al., 2000).

Predictions of novel drug design for ligands of the NMDA receptor adopted from molecular modelling should be interpreted with caution since the molecular architecture of natively expressed NMDA receptors remains elusive. Additional subunits, cytoskeletal proteins, correct protein folding and post-translational modifications may be required to reconstitute receptors with all of the structural characteristics of native NMDA receptors (Sucher et al., 1996).

These findings presented in this project will form the basis of further novel investigations into the development of NMDA receptor subtype-selective ligands. As is the case with research, the end of one project paves the way for the beginning of another.
Table 4.1: Summary of the binding parameters determined by saturation radioligand binding to recombinant NR-1-1a subunits

<table>
<thead>
<tr>
<th>Radioligand</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[$^3$H] MDL105,519</td>
<td>3.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>(This thesis)</td>
</tr>
<tr>
<td>[$^3$H] MDL105,519</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(Siegel et al., 1996)</td>
</tr>
<tr>
<td>[$^3$H] 5,7 DCKA</td>
<td>41 ± 11</td>
</tr>
<tr>
<td></td>
<td>(Lynch et al., 1993)</td>
</tr>
<tr>
<td>[$^3$H] L689,560</td>
<td>3.29 (2.94, 3.67)</td>
</tr>
<tr>
<td></td>
<td>(Grimwood et al., 1995)</td>
</tr>
</tbody>
</table>
Table 4.2: Summary of the binding parameters determined from either saturation or competition radioligand binding to recombinant NR1-1a/NR2A or *native membranes

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ or $K_d$ (nM)</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>$K_i = 3900 \pm 1000$ (This thesis)</td>
<td>$1.0 \pm 0.1$ (This thesis)</td>
</tr>
<tr>
<td>Glycine</td>
<td>*$K_i = 580 \pm 370$ (Chazot et al., 1998)</td>
<td>*$0.8 \pm 0.1$ (Chazot et al., 1998)</td>
</tr>
<tr>
<td>5,7 DCKA</td>
<td>$K_i = 50 \pm 10$ (This thesis)</td>
<td>$0.9 \pm 0.2$ (This thesis)</td>
</tr>
<tr>
<td>5,7 DCKA</td>
<td>$K_d = 59 \pm 8$ (Laurie and Seeburg, 1994)</td>
<td>1.0        (Laurie and Seeburg, 1994)</td>
</tr>
<tr>
<td>MDL105,519</td>
<td>$K_d = 1.9 \pm 1.0$ (This thesis)</td>
<td>1.0        (This thesis)</td>
</tr>
<tr>
<td>MDL105,519</td>
<td>*$K_i = 4.3 \pm 0.7$ (Baron et al., 1996)</td>
<td>*$1.2 \pm 0.1$ (Baron et al., 1996)</td>
</tr>
<tr>
<td>L701,324</td>
<td>$K_i = 4.2 \pm 1.1$ (This thesis)</td>
<td>1.0 $\pm 0.2$ (This thesis)</td>
</tr>
<tr>
<td>L701,324</td>
<td>*$K_i = 2.5 \pm 0.2$ (Parsons et al., 1997)</td>
<td>*$1.1 \pm 0.1$ (Parsons et al., 1997)</td>
</tr>
<tr>
<td>L689,560</td>
<td>$K_1 = 6.0 \pm 1.2$</td>
<td>$0.6 \pm 0.2$</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td></td>
<td>(This thesis)</td>
<td>(This thesis)</td>
</tr>
<tr>
<td>L689,560</td>
<td><em>$K_1 = 3$</em></td>
<td><em>1.0</em></td>
</tr>
<tr>
<td></td>
<td>(Grimwood et al., 1995)</td>
<td>(Grimwood et al., 1995)</td>
</tr>
<tr>
<td>L689,560</td>
<td><em>$K_1 = 4.4 \pm 0.38$</em></td>
<td><em>1.1 \pm 0.1</em></td>
</tr>
<tr>
<td></td>
<td>(Parsons et al., 1997)</td>
<td>(Parsons et al., 1997)</td>
</tr>
</tbody>
</table>
Figure 4.1: Comparison of the chemical structures of glycine site antagonists of the NMDA receptor

Detailed analysis of the chemical structures of MDL105,519 with both GV150,526A and GV196,771A revealed distinct structural differences between the ligands. For example, both GV150,526A and GV196,771A exist as trans stereoisomers with phenylamide moieties (shown in boxes) conjugated to the phenyl ring. In addition both compounds have six spacer carbon atoms (●) separating the pyrrole group from the phenyl ring. In contrast, MDL105,519 is a cis stereoisomer with four spacer carbon atoms (●) between the indole nucleus and the phenyl group.
Figure 4.2: Chemical structures of glycine site ligands of the NMDA receptor

The filled areas show the molecule determinants shared by all the glycine site antagonists; the hatched areas show the structural moieties shared between those antagonists that result in complex inhibition curves with regard to displacement of $[^3]$H] MDL105,519 radioligand binding.
Glycine site ligands may be subdivided into two pharmacological classes, i.e. those that show simple competitive behaviour with regard to inhibition of $[^3H] \text{MDL105,519}$ radioligand binding to heteromeric NMDA complexes and those that result in complex inhibition curves.
APPENDIX

PRINCIPLES OF RADIOLIGAND BINDING STUDIES

Radioligand binding studies provide vast information concerning a receptors pharmacological characteristics with respect to its ligand affinity, selectivity, structure activity relationship and an assessment of receptor density. The goal of radioligand binding studies is to investigate the physicochemical interaction between a single population of homogenous binding sites, R, and the radioligand, L, in a fully reversible reaction at equilibrium. The respective interaction behaves via the law of mass action as the rate of reaction is driven by the quantities (mass) of reactants in each side of the equilibrium. Such a biomolecular interaction is represented by equation 1.

\[
R + L \leftrightarrow RL
\]

(1)

Where;

- \(k_{+1}\) = association rate constant
- \(k_{-1}\) = dissociation rate constant
- RL = radioligand receptor complex

Implicit to the above equation, several criteria need to be fulfilled before physical parameters are quantified i.e., affinity and maximal number of binding sites. Firstly, both the receptor and radioligand should be unaltered during the experimental procedure. Secondly, it should be assumed that all receptors in the membrane preparation are equally
accessible to the radioligand. In the context of a radioligand binding study, the term receptor is synonymous for the term 'binding site'. The definition of a binding site in radioligand binding assays is characterised by the following requirements including that the receptor population should have a finite number of specific binding sites thus demonstrating saturable binding and that binding sites labelled by the radioligand should exhibit specificity and stereo-specificity. The biologically active (+/-) stereo-isomer forms of a ligand should be more potent than their non-biologically active counterparts and, should displace the bound radioligand with lower affinity. Moreover, the binding of the ligand to the binding site should be fully reversible. However, demonstrating saturability, selectivity and reversibility are insufficient evidence to ensure that the observed binding is an actual interaction between the radioligand and the receptor of interest. This was exemplified with [125I] insulin which was shown to bind to talc and fulfilled all the above criteria (Cuatrecasas and Hollenberg, 1975). Therefore, an additional requirement that should also be maintained is that the affinity of the radioligand for the receptor preparation should be appropriate to the concentration range in which the ligand elicits a biological response in vivo.

The choice of the radioligand depends on several considerations depending on whether an agonist or antagonist is desirable, and whether a tritiated or iodinated ligand is the most suitable. Iodinated ligands have higher specific radioactivity compared to [3H] ligands usually in the range of 16250 Ci/mmol versus 70 Ci/mmol. However, iodination of a ligand usually alters the chemical structure of the compound and iodinated radioligands have relatively short half lives, 8 days for [131I] and 60 days for [125I]. In contrast, [3H] ligands
have longer radioactive half lives of 12.5 years and importantly, they do not alternate the chemical structure of the ligand. However, tritiated radioligands undergo low energy beta emissions thus necessitating the use of scintillation spectroscopy to quantify the bound radioligand complex.

The radiolabelled ligand employed in radioligand binding assays should satisfy the following considerations including that it is biologically active, chemically pure and stable within the assay. In addition, the labeled ligand has to maintain the same binding characteristics as its unlabelled form. Furthermore, the rate of dissociation of the radioligand from the receptor sites must be slow enough to employ a separation technique to select for the unbound ligand without disturbing the equilibrium.

The method undertaken to separate free ligand from bound ligand is dependent on the dissociation rate constant of the radioligand. A pivotal factor governing the rate of dissociation of the radioligand is its affinity for the binding site. Low affinity radioligands usually have fast dissociation constants. Conversely, high affinity radioligands have slower dissociation constants. Several techniques have been developed to separate the unbound ligand such as centrifugation, equilibrium dialysis, gel filtration and filtration. The filtration method is by far the most popular technique employed in radioligand binding assays due to its ease of use, reproducibly of the results and low non-specific background. The two essential factors that should be satisfied in order to minimize any dissociation of radioligand from the receptor binding site in this technique are that the separation should be accomplished within 10 sec and radioligand should have a $k_\text{i}$ of $< 0.4 \times 10^{-2} \text{sec}^{-1}$. Ligands with $k_\text{i} > 0.4 \times 10^{-2} \text{sec}^{-1}$ usually employ a centrifugation assay for separation of the receptor-
ligand complex since equilibrium is maintained during the separation procedure. However, a loss of bound ligand only may occur due to the washing of the pellet which could potentially disturb the equilibrium of the reaction (equation 1).

Before embarking on any study of receptor physiology using the radioligand binding technique, it is imperative that each of these criteria has been rigorously fulfilled.

As stated above, the radioligand interaction with the binding site is the parameter of interest however, the radioligand also labels sites other than the specific receptor binding site. For example, glass surfaces, biological membranes and filters used in the separation procedure. This binding is referred to as non-specific radioligand binding. This feature of binding assays obviously necessitates the definition of specific radioligand binding and its separation from non-specific binding. The characteristic features of non-specific binding which allow it be distinguished from specific binding include non-saturability and low affinity. In practice, non-specific binding can be determined using a concentration of an unlabelled ligand which should ideally be chemically dissimilar to the radioligand, but must be specific for the same receptor binding site to block fully the binding sites of interest. This will reduce the possibility of displacement of the radioligand from non-specific binding sites.

Non-specific binding is defined in radioligand binding assays by incubating the reaction in parallel set of tubes containing either the radioligand or radioligand in the presence of unlabelled competitor used to define nonspecific-binding. The non-specific binding is calculated by subtracting the total radioligand binding from the binding of the labelled ligand in the presence of the unlabelled ligand defining non-specific binding. The
assumption underlining this method is that the level of non-specific binding is similar in both the total tubes and the tubes containing the labelled and unlabelled ligand. However, non-specific binding is directly proportional to the free radioligand concentration which is not the same in the two sets of tubes. This discrepancy between the free ligand in the total and non-specific binding incubation tubes becomes negligible if the total binding is less than 10% of the free ligand concentration i.e., if radioligand depletion is kept to a minimum. This latter consideration is a principal assumption used in the derivation of equations for the analysis of radioligand binding data.

Additionally, the ratio of specific to non-specific radioligand binding to neurotransmitter receptors can be increased by either sonification, freeze thawing or homogenisation of the membranes, to rupture the synaptic vesicles. This ensues that any endogenous neurotransmitters are removed which may interfere in the radioligand binding assay.

There are three major types of radioligand binding assays that can be performed depending on the information required including kinetic, saturation and inhibition experiments. Kinetic studies monitor the binding of a fixed concentration of radioligand to the physiologically relevant receptor over time allowing the determination of both the $k_{on}$ and the $k_{1}$ of the radioligand. These parameters allow the calculation of both the time taken to attain equilibrium ($k_{on}$) and equilibrium dissociation constant ($K_D$) of the reaction, as shown in equation 2.
For saturation experiments, the theoretical model used is one of equilibrium. As stated above, one of the salient features of radioligand binding to the physiologically relevant receptor, is that the binding sites should be saturable. To assess saturability the characteristics of binding as a function of increasing concentrations of radioligand is determined. If the bound radioactivity represents the binding of a radioligand to a saturable receptor population, the plot of bound radioligand versus [radioligand] will yield a rectangular hyperbola (section 2.2.21.2). Both the $K_D$ of the ligand and maximum number of binding sites, $B_{\text{max}}$, can be determined from the saturation isotherm. The $B_{\text{max}}$ value is obtained from the specific binding curve that asymptotically approaches a value parallel to the x-axis. The $K_D$ is calculated at the radioligand concentration equivalent to $B_{\text{max}}/2$. However, $B_{\text{max}}$ will be attained at infinite concentrations of the radioligand thus in practice these values are approached but never observed.

In reality, saturation of the receptor binding sites with the radioligand is rarely obtained although a plateau may be observed on the saturation isotherm. Transforming the radioligand binding data to a plot of bound versus $\log_{10}$ [radioligand], will disclose a truncation of the isotherm if saturation is not apparent.

Historically, saturation radioligand binding data was linearised which allowed the data to be fit by hand. The plot of bound/free (B/F) radioligand versus bound (B) radioligand allowed the determination of the $K_D$ from the negative reciprocal of the slope and $B_{\text{max}}$. 

$$\frac{k - 1}{k + 1} = K_D \quad (2)$$
from the x-axis intercept. This plot is commonly referred to as the Scatchard transformation. However, the Rosenthal plot was introduced to deal with situations where the receptor concentration is unknown, as typically is the case when binding to impure receptor preparations (Rosenthal, 1967). This is a pivotal assumption in the derivation of the Rosenthal transformation. In contrast, the Scatchard analysis assumes the concentration of the binding sites. Therefore, strictly the plot of B/F versus B is a Rosenthal plot and not a Scatchard transformation. Determination of the binding parameters from the linear Rosenthal transformation of the saturation data generally confers unreliable determinants. Technical limitations often prevent evaluating bound receptor-ligand complex over a broader range of radioligand concentrations as a result the extrapolation onto the x-axis is in considerable error. Another drawback of linear regression is that the saturation data is transformed, i.e. bound radioligand is divided through free radioligand concentration which distorts the experimental errors of bound. This is because any errors made in the determination of the free radioligand concentration will be repeated in the values calculated for B/F. Furthermore, linear transformation of the saturation data compresses both the x and y-axis ends of the regression which gives a false interpretation that the plot is linear when in reality it may deviate substantially from linearity. Furthermore by fitting linear regression, experimental outliers are often ignored which may have a great influence on the final values of Bmax.

A far superior method in determining the $K_D$ and $B_{max}$ values is the direct analysis of the untransformed saturation radioligand binding data by non-linear regression. Computerised non-linear least squares regression analysis fits the saturation data objectively by weighting
the error of measurement at each data point.

A linear Rosenthal plot implies a non-interacting homogenous receptor population. Deviations from linearity may be the result of several different phenomena that include impurity and/or stability of the radioligand; binding to a heterogeneous population of receptors which have different affinities for the ligand; the existence of two binding sites within a receptor with different affinities for the ligand; different states of an apparently single binding site and co-operativity. Alternatively, the apparent deviation may be due to radioligand binding artefacts in the assay. For example, incomplete equilibration with the radioligand, over or underestimation of non-specific binding. Accordingly, to detect two receptor subtypes from a saturation curve the K_D values must be sufficiently different and the radioligand concentration used should encompass the K_D values.

Alternatively, to determine whether the ligand-receptor interaction follows a simple bimolecular reaction (equation 1), the saturation data could be transformed to a Hill plot as shown in equation 3.

\[
\frac{n_H \log L - n_H \log K_{0.5}}{B_{\text{max}} - B} = \log \frac{B}{B_{\text{max}} - B} \tag{3}
\]

Where;

B = specific bound radioligand

B_{\text{max}} = maximal number of binding sites
The Hill coefficient, $n_H$, is obtained as the slope of the linear regression. If the ligand is binding to a single species of receptor via a simple reversible bimolecular reaction, $n_H = 1$. However, $n_H > 1$ is indicative of positive co-operativity i.e. there is an increase in affinity of the overall receptor population as occupancy of the receptor increases with the radioligand. In contrast, negative co-operativity which describes the decrease in affinity for the next ligand molecule by the first bound ligand to the receptor site is characterised by $n_H < 1$.

The specificity with which the radioligand interacts with the binding sites is determined by competition radioligand binding assays. These experiments are performed by incubating the radioligand and receptor which are both held at a constant concentration with increasing concentrations of the unlabelled displacer. Competition data are usually displayed as percentage of specific radioligand binding versus $\log_{10} [\text{unlabelled competitor}]$. The concentration of the competitor that effectively competes for 50% of the specific radioligand binding is termed the IC$_{50}$ value. This parameter is dependent upon the radioligand concentration employed in the binding assay. To negate the influence of the radioligand concentration on the affinity of the competitor, the absolute inhibition constant, $K_i$, is calculated. The $K_i$ is the concentration of the unlabelled ligand which occupies 50% of the receptor sites if no radioligand were present. It is determined from the Cheng-Prusoff
equation using the respective IC$_{50}$ value (section 2.2.21.1).

Displacement radioligand binding experiments are very useful in indirectly characterising ligands which have a low affinity for a binding site. Also, the heterogeneity of a receptor preparation can be demonstrated according to the characteristic binding profiles of different receptors or receptor subtypes. For example, if the labelled and unlabelled ligand compete for a single binding site, the competitive binding curve will have a shape determined by the law of mass action (see equation 1). In this case, the descend from 90% to 10% specific radioligand binding on the competition curve will cover two log units (100-fold change in concentration). Complexity of the competition reaction is characterised by shallow displacement curves, where the descend from 90% to 10% specific radioligand binding on the competition curve will be > than 2 log units.

In summary, by fulfilling the considerations outlined above and careful interpretation of the radioligand binding results, this technique can prove to be an extremely powerful tool in the elucidation and characterisation of receptors subtypes.
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