NMDA Receptor Subtypes Expressed in Mammalian Cells: A Molecular Characterisation

by

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ABSTRACT

The N-methyl-D-aspartate (NMDA) subtypes of the excitatory glutamate receptors of the mammalian brain are fast-acting, ligand-gated ion channels. These receptors have been implicated in long term potentiation, synaptic plasticity and neurodegeneration. The NMDA receptor channel is highly permeable to Ca$^{2+}$ and NMDA receptor activity can be modulated by several classes of compounds which include neurosteroids, Mg$^{2+}$, Zn$^{2+}$, H$^{+}$ and polyamines. There are five genes encoding NMDA receptor subunits, NR1 and NR2A-2D. These genes are classified according to the amino acid homologies of their respective products. It is believed that native NMDA receptors are composed of an NR1 together with (a) NR2 subunit(s) in unknown ratios. It was the aim of this project to elucidate the subunit complements of native cerebellar NMDA receptors. The NR2C mRNA is abundantly expressed in the cerebellum therefore NR1 and NR2C clones were coexpressed transiently in human embryonic kidney (HEK) 293 cells. The recombinant receptor was characterised by both immunoblotting and $[^3]$H]MK801 radioligand binding assays and the affinity compared to that of native NMDA receptors expressed in the cerebellum. The NR1/NR2C receptor had an affinity, $K_D$, for $[^3]$H]MK801 = 345 ± 158 nM compared to 22 ± 9 nM for the wild-type. However, cotransfection with NR1, NR2C and NR2A clones using a defined DNA ratio for transfection yielded a $K_D = 22 ± 5$ nM. A detailed characterisation of the pharmacology of this binding site showed that the correlation coefficient for the affinities of a series of compounds between NR1/NR2A/NR2C and native cerebellar receptors was $r = 0.992$. Further studies showed that the NR1, NR2A and NR2C ratios used for transfection could influence the pharmacological profile of the recombinant receptor. The radioligand binding studies to the cloned NMDA receptor subtypes suggested that the determinant for the high affinity MK801 binding resided in the NR2A subunit. This has been investigated further by site-directed mutagenesis of the NR2C subunit within the transmembrane, TM2, region in conjunction with $[^3]$H]MK801 radioligand binding and cell cytotoxicity assays.
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ABBREVIATIONS

ACPD  1-aminocyclopentane-1,3-dicarboxylate
AMPA  α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AP5    D-2-Amino-5-phosphonopentanoic acid
AP7    D-2-Amino-7-phosphoheptanoic acid
APS    Ammonium persulphate
ATP    Adenosine triphosphate
ATX    Argiotoxin$_{36}$
B$_{max}$ Maximum receptor density
bp     Base pairs
BSA    Bovine serum albumin
cDNA   Complementary DNA
CCG    2-(carboxycyclopropyl) glycine
CGP 37849 (E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid
CGP 39653 (E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid
CGS 19755 Cis-4-phosphonomethylpiperidine-2-carboxylic acid
CHO    Chinese hamster ovary
CIP    Calf intestinal alkaline phosphatase
7-CK   7-Chlorokynurenic acid
CMV    Cytomegalovirus
CNQX   6-Cyano-7-nitroquinoxaline-2,3-dione
CNS    Central Nervous System
CPP    (D)-3-(2-Carboxypiprazin-4-yl)propyl-1-phosphonic acid
CPPene (E)-R-(3-Phosophonopro-2-enyl)piperazine-2-carboxylic acid
dATP   Deoxy-adenosine-5′-triphosphate
dATPαS Deoxy-adenosine-5′-[αS]thiotriphosphate
dCTP   Deoxy-cytosine-5′-triphosphate
dCTPαS Deoxy-cytidine-5′-[αS]thiotriphosphate
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<tr>
<td>ddNTPs</td>
<td>Dideoxynucleoside-5’-triphosphates</td>
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<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy-guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>DKA</td>
<td>5,7-Dichlorokynurenic acid</td>
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<tr>
<td>DMEM/F-12</td>
<td>Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham</td>
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<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<td>DNA</td>
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<td>DNQX</td>
<td>6,7-Dinitroquinoxaline-2,3-dione</td>
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<td>DTT</td>
<td>Dithiolthreitol</td>
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<tr>
<td>dTTP</td>
<td>Deoxy-thymosine-5’-triphosphate</td>
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<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
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</tr>
<tr>
<td>EGTA</td>
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</tr>
<tr>
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<td>Foetal calf serum</td>
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<td>γ-Aminobutyric acid</td>
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<td>GABA_A,R</td>
<td>Ionotropic γ-aminobutyric acid receptor</td>
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<td>GluR</td>
<td>Glutamate receptor subunit</td>
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<td>G-protein</td>
<td>Guanine nucleotide-binding regulatory protein</td>
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<td>GYKI 52466</td>
<td>1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine</td>
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<td>HA-966</td>
<td>1-Hydroxy-3-amino-pyrrolidine-2</td>
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<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>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
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<tr>
<td>I/V</td>
<td>Current-voltage</td>
</tr>
<tr>
<td>KA</td>
<td>Kainate receptor subunit</td>
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<tr>
<td>K_D</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>K_I</td>
<td>Inhibition constant</td>
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<td>L-687414</td>
<td>(R)-(+) cis-β-methyl-HA-966</td>
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<td>L-689560</td>
<td>(±)-Trans-2-carboxy-5,7-dichlorotetrahydroquinoline-4-phenylurea</td>
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<td>L-2-amino-3-phosphonopropionic acid</td>
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<td>M</td>
<td>Membrane region</td>
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<tr>
<td>MAP</td>
<td>Multiple antigen peptide</td>
</tr>
<tr>
<td>MCPG</td>
<td>(+)-α-Methyl-4-carboxyphenylglycine</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MK801</td>
<td>5-Methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propane-sulphonic acid</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular mass</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NBQX</td>
<td>6-Nitro-7-sulphamoylbenzoquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NR</td>
<td>NMDA receptor subunit</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ori</td>
<td>Origin of replication</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMFS</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>RF DNA</td>
<td>Replicative form DNA</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAase</td>
<td>Ribonuclease A</td>
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<tr>
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<td>Post-synaptic density protein 95</td>
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<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SKF-10,047</td>
<td>N-Allylnormetazocine</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>sv40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TB media</td>
<td>Terrific broth media</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCP</td>
<td>Tenoyclidine</td>
</tr>
<tr>
<td>TEMED</td>
<td>(NNN'N')-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)methylamine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-Bromo-4-chloro-3-indolyl-(\beta)-D-galactoside</td>
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ACKNOWLEDGMENTS

Many thanks to my supervisor Professor F. Anne Stephenson for allowing me to work and study for this Ph.D. in her laboratory. Her help and advice throughout was much valued.

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Most especial thanks to my family, David, Lynette and Rebecca for helping me settle into life in the BIG city, and my parents for their unwavering support throughout.
CHAPTER 1

General Introduction
1.1 FOREWORD

The brain is the inner universe through which all external happenings are perceived. The content and structure of the human brain defines the individual, it is the most complex organ of the body with the most complex of functions. The human brain contains at least $10^{12}$ cells, a tenth of them neurones. Each neurone can form 1000 synaptic connections and receive yet more.

Research upon the brain and neuroscience has its basis in the last century, in the demonstration by Ramon y Cajal that the neurone was the basic functioning unit and in Sherrington's proposal of 'specialized junctional complexes' between neurones, which he termed synapses. The first suggestion of chemical transmission between nerves was made by Elliot in 1904. This idea was supported by the pharmacological studies of Dale in 1914. Previously, in 1909, Langley and Dale had suggested that 'receptive substances' must exist on the surface membranes of excitable cells (reviewed in Siegel et al., 1994). However, the first evidence of chemical transmission came from Loewi in 1921. It is interesting that the design of the experiment came from a dream:

"The next night, at 3.00 am, the idea returned. It was the design of an experiment to determine whether or not the hypothesis of chemical transmission that I had uttered seventeen years ago was correct. I got up immediately, went to the laboratory, and performed a simple experiment on a frog heart according to the nocturnal design. . . . the hearts of two frogs were isolated, the first with its nerves, the second without, both hearts were attached to Straub cannulas filled with a little Ringer solution. The vagus nerve of the first heart was stimulated for a few minutes. Then the Ringer solution that had been in the first heart during the stimulation of the vagus nerve was transferred to the second heart. . . . These results unequivocally proved that the nerves do not influence the heart directly but liberate from their terminals specific chemical substances which, in their turn, cause the well-known modifications of the function of the heart characteristic of the stimulation of its nerves."

(reviewed in McGeer et al., 1978)
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Loewi termed this substance 'Vagusstoff'. It was later identified as acetylcholine. Since then a large number of other neurotransmitters have been discovered. In the central nervous system (CNS) the major inhibitory neurotransmitters are γ-aminobutyric acid (GABA) and glycine, whereas L-glutamate is the major excitatory neurotransmitter. The process of chemically-mediated synaptic transmission involves a number of processes. The neurotransmitter (or its precursor) needs to be synthesised and stored at the presynaptic terminal. Upon stimulation of the presynaptic neurone, the neurotransmitter is released into the synaptic cleft (Figure 1.1). The main mechanism of release is Ca\(^{2+}\)-dependent. Ca\(^{2+}\) entry leads to the fusion and exocytosis of the neurotransmitter-containing vesicles. The mechanism of action of Ca\(^{2+}\) is unclear, but probably involves activating a protein kinase, which initiates an event cascade. Upon release into the synaptic cleft, the neurotransmitter recognizes and binds specifically to the postsynaptic receptor proteins. The resulting conformational change will lead to a change in ion flux across the membrane, or the activation of secondary messenger systems. Following interaction with the receptor, the neurotransmitter is removed from the synaptic cleft. This can occur through uptake mechanisms, transporting the neurotransmitter into nerve terminals or glial cells, e.g. for glutamate, GABA or dopamine, or by extracellular enzymes which degrade the neurotransmitter, such as acetylcholinesterase which converts acetylcholine to acetate and choline (reviewed in Siegel et al., 1994).

1.2 GLUTAMATE AS A NEUROTRANSMITTER

Glutamate is ubiquitously distributed throughout the brain. Moreover, the concentration of glutamate is considerably higher than any other amino acid in the brain. However, glutamate does not cross the blood-brain barrier and is synthesised within the brain. The Krebs cycle in mitochondria produces α-ketoglutarate. This can be used to synthesize glutamate by transamination or via
the enzyme glutamic acid dehydrogenase. Glutamate can also be produced from glutamine via glutaminase. Glial cells take up released glutamate and convert it to glutamine via glutamine synthetase. The glutamine is then transported to the neuron (Siegel et al., 1994; Schousboe et al., 1993).

The first evidence for glutamate as a neurotransmitter came in 1954 when Hayashi demonstrated the convulsive effects of L-glutamate and L-aspartate in the mammalian brain by direct injection into the cerebral cortex (Hayashi, 1954). Later that decade, Curtis et al. (1959) showed that L-glutamate and L-aspartate had an excitatory and depolarising effect on single neurones in the central nervous system (CNS). In 1971, Wofsey et al. (1971) demonstrated a high-affinity uptake system for glutamic and aspartic acids. Further evidence also supported the role of L-glutamate as a neurotransmitter; it was pre-synaptically localized in specific neurones; physiological stimulation specifically released glutamate in a Ca\(^{2+}\)-dependent manner and in concentrations high enough to elicit a post-synaptic response; glutamate showed identity of action with the naturally occurring neurotransmitter, including its response to antagonists (reviewed in Fonnum 1984). As a result it was accepted that L-glutamate was the major excitatory neurotransmitter in the mammalian CNS. Studies with agonists and antagonists showed that L-glutamate acted at a range of receptor types (reviewed in Mori and Mishina, 1995; Bettler and Mulle, 1995; Pin and Duvoisin, 1995). Many functions of L-glutamate have been elucidated; it mediates the majority of fast excitatory synaptic transmission, it plays important roles in synaptic plasticity and memory formation (Bliss and Collingridge, 1993) and in the early development of the nervous system (Mattson et al., 1988). Glutamate also has a pathological role in neuronal degeneration and neuronal cell death (Meldrum and Garthwaite, 1990).
Figure 1.1 A schematic diagram of a typical glutamergic synapse

PSD-95 refers to the post-synaptic density protein discovered to interact with the NMDA receptor subunits by Korneau et al. (1995).
1.3 THE GLUTAMATE RECEPTORS

The multiple functions of L-glutamate are mediated through its receptors. On the basis of pharmacological, biochemical and electrophysiological studies, the mammalian glutamate receptors have been divided into two major groups, termed the ionotrophic and metabotropic glutamate receptors (mGluR). The ionotrophic receptors contain a glutamate-gated, cation-specific ion channel. Metabotropic glutamate receptors are linked to GTP-binding proteins (G-proteins), and modulate the production of intracellular messengers. The ionotrophic glutamate receptors have been further subdivided into N-methyl-D-aspartate (NMDA) receptors and the non-NMDA receptors. The non-NMDA receptors are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors and kainate receptors, according to their selective agonist. The glutamate receptor subtypes were distinguished pharmacologically and electrophysiologically prior to the glutamate receptor genes being cloned. Advances in the molecular biology of the mammalian glutamate receptors have upheld these broad distinctions. However, the cloning of multiple genes for each glutamate receptor subtype has added a new level of complexity.

1.3.1 Molecular Pharmacology of the NMDA Receptor

The NMDA receptors were defined by their activation with the selective agonist NMDA. This agonist was originally synthesised for the investigation of structure-activity relationships of L-glutamic acid and L-aspartic acid by Watkins (1962) and was found to be a 1000-fold more potent in eliciting an excitatory response than L-glutamate.

The NMDA receptor has a number of unique properties which are fundamental to its physiological roles. The gating of the NMDA receptor channel is unique in being both ligand- and voltage-dependent. The activation of the
CHAPTER 1

Glutamate Site Agonists

L-Glutamate

L-Aspartate

NMDA

Competitive Antagonists

DKA

D-AP5

D-CPP

Glycine Site Agonists

Glycine

D-Serine

Glycine Site Partial Agonist

(+)-HA-966

Channel Binding Non-Competitive Antagonists

TCP

Ketamine

MK801

Figure 1.2 Structures of agonists and antagonists of the NMDA receptor
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NMDA receptor requires the presence of the agonist (L-glutamate) and the co-agonist, glycine (Kleckner and Dingledine, 1988) and release of the voltage-dependent block. The voltage-dependence is caused by Mg$^{2+}$ binding within the ion channel (Mayer et al., 1984; Nowak et al., 1984). The integral ion channel of NMDA receptors was found to be highly permeable to K$^+$, Na$^+$ and Ca$^{2+}$ (Ascher and Nowak, 1988). A Zn$^{2+}$ modulatory site was also found, which, in contrast to the Mg$^{2+}$ site, was relatively voltage-insensitive (Peters et al., 1987; Westbrook and Mayer, 1987). The NMDA receptor channel also contains a binding site for dissociative anaesthetics, such as phencyclidine (PCP), ketamine and 5-methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine ((+)-MK801) (Anis et al., 1983; Honey et al., 1985; Wong et al., 1986). Further modulatory sites for polyamines, neurosteroids and ethanol have been described (Williams et al., 1989; Bowlby, 1993; Lovinger et al., 1989). The NMDA receptor may also be modulated in vivo by H$^+$ and redox reagents (Traynelis and Cull-Candy, 1990; Aizenman et al., 1989). The modulatory sites of the NMDA receptor are summarized in Figure 1.3, and are discussed in more detail below.

1.3.1.1 The glutamate modulatory site

Following the discovery of the convulsive and excitatory effects of L-glutamate and L-aspartate a study was made of the actions of related compounds. It was found that N-methyl-D-aspartic acid was considerably more potent than the original compounds, as was D-homocysteic acid (Watkins, 1962). Recently discovered agonists at the NMDA receptor glutamate site include the 2S,3R,4S derivative of 2-(carboxycyclopropyl)glycine (CCG) and (tetrazol-5-yl)glycine (Shinozaki et al., 1989; Schoepp et al., 1991). Both of these compounds are more potent agonists than NMDA and their restricted conformation suggests that L-glutamate binds to NMDA receptors in a folded conformation, different from that required for AMPA and kainate receptor activation (Bigge, 1993).

In the early 1980s the competitive antagonists D-2-amino-5-
phosphopentanoic acid (AP5) and D-2-amino-7-phosphoheptanoic acid (AP7) were described (Davies et al., 1981; Perkins et al., 1981). Many recently developed competitive antagonists for the glutamate binding site are cyclic derivatives of AP5 or AP7. Examples include (D)-3-(2-carboxypiperazin-4-yl)propyl-phosphonic acid (CPP) and (E)-R-(3-phosphonopro-2-enyl)piperazine-2-carboxylic acid (CPPene), both derived from AP7 and cis-4-phosphonomethylpiperidine-2-carboxylic acid (CGS 19755) and (E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP 37849) both derived from AP5 (Bigge, 1993).

1.3.1.2 The glycine modulatory site

Johnson and Ascher (1987) found that sub-micromolar concentrations of glycine potentiated the response to NMDA in neuronal cultures. This potentiation has now been shown in a wide variety of cultures and preparations (reviewed in Thomson 1990). Experiments on Xenopus oocytes injected with rat brain mRNA by Kleckner and Dingledine (1988) demonstrated that glycine was absolutely required for NMDA receptor channel opening. This potentiation by glycine was not antagonised by strychnine. Therefore this action is distinct from the classical inhibitory response of the strychnine-sensitive glycine receptor (Hamill et al., 1983). Parallel studies showed that glycine facilitated the binding of the PCP site ligands (see 1.4.1.4) to well-washed brain membranes (Loo et al., 1986; Snell et al., 1987; Reynolds et al., 1988). Further investigation showed that the NMDA-sensitive $[^3]$H]glutamate binding sites had a very similar autoradiographic distribution to the strychnine-insensitive $[^3]$H]glycine binding sites on rat brain membranes (Bowery, 1987). Fletcher and Lodge (1987) demonstrated that NMDA receptor antagonism induced by 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) could be reversed by the co-administration of glycine. HA-966 had been shown to displace $[^3]$H]glycine binding, but is now accepted as a low-efficacy partial agonist for the glycine site (Foster and Kemp 1989).
A number of glycine site antagonists have now been developed, many are derivatives of kynurenic acid, an example is 5,7-dichlorokynurenic acid (DKA), see Figure 1.2. The most potent antagonist to date is trans-2-carboxy-5,7-dichlorotetrahydroquinoline-4-phenylurea (L-689,560) (Foster et al., 1992). This was also derived by modification of the basic heterocyclic ring of kynurenic acid. However, while highly active in vitro, many of these kynurenic acid-derived glycine site antagonists are only weakly active in vivo, when given systemically, due to poor CNS availability.

1.3.1.3 The divalent cation binding sites

The unique property of NMDA receptors is the voltage-dependent Mg\(^{2+}\) block. This means that for channel opening not only is binding of the co-agonists required, but membrane depolarization is also necessary. This is fundamental to the physiological roles of the NMDA receptor (see 1.5). The antagonistic effect of physiological concentrations of extracellular Mg\(^{2+}\) at NMDA receptors was demonstrated by Ault et al. (1980). Further work in the early 1980s showed that this Mg\(^{2+}\) block was voltage-sensitive (Nowak et al., 1984; Mayer et al., 1984). Maximum block occurred at approximately -80 mV and membrane depolarization caused a gradual relief of the Mg\(^{2+}\) blockade. Reports have suggested that the amplitude of the block for a given cell type may depend on the age of the animal (Ben-Ari et al., 1988; Kleckner and Dingledine, 1991). This maybe due to post-translational modification or a developmental subunit 'switch' (Chen and Huang, 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992).

The initial work of Nowak et al. (1984) also suggested an intracellular Mg\(^{2+}\)-dependent block. In contrast to the extracellular block by Mg\(^{2+}\), the intracellular Mg\(^{2+}\)-dependent block increased with depolarisation. This was characterized at the single channel level (Johnson and Ascher, 1990). At intracellular Mg\(^{2+}\) concentrations of 1 mM, the block was detectable only at positive potentials, while at 10 mM intracellular Mg\(^{2+}\) the block was detectable...
CHAPTER 1

at negative potentials. Intracellular Mg$^{2+}$ dissociated more rapidly than did extracellular Mg$^{2+}$, suggesting that the blocking effects of intracellular and extracellular Mg$^{2+}$ occurred at distinct sites.

Other divalent cations also modulate the NMDA receptor channel. Electrophysiological studies on cultured neurones demonstrated selective and non-competitive antagonism by Zn$^{2+}$ (Peters et al., 1987; Westbrook and Mayer, 1987). This effect was also seen in TCP binding studies (Reynolds and Miller, 1989b) and neurotoxicity studies (Peters et al., 1987). The site preferentially bound Zn$^{2+}$, however Hg$^{2+}$ and Cd$^{2+}$ were also recognised (Westbrook and Mayer, 1987). This action of Zn$^{2+}$ was not voltage-dependent but was due to reduced channel open time (Mayer et al., 1988). Thus Zn$^{2+}$ was acting at a site distinct from that of Mg$^{2+}$. Radioligand binding studies showed that Zn$^{2+}$ and Cd$^{2+}$ inhibited the binding of $[^{3}H]$glutamate to the NMDA receptor, in contrast to Mg$^{2+}$ and Ca$^{2+}$ (Monahan and Michel, 1987). Recent studies have also demonstrated a Ca$^{2+}$ binding site within the NMDA receptor pore (Burnashev et al., 1995).

1.3.1.4 The PCP binding site

In the early 1980s it was shown that the dissociative anaesthetics, ketamine and PCP, selectively reduced NMDA-induced excitation of mammalian neurones (Anis et al., 1983). Honey et al. (1985) demonstrated these compounds gave a use- and voltage-dependent block of the NMDA receptor. This suggested that they were binding within the open ion channel and acting as non-competitive antagonists. Due to the proposed location of the binding site, it was thought that glutamate and glycine binding was required for the receptor to be in the appropriate configuration for the PCP site ligands to bind (Loo et al., 1986; Ransom and Stec, 1988). The anticonvulsant MK801 was also shown to be a potent NMDA antagonist at the PCP site - PCP and ketamine were able to compete for $[^{3}H]$MK801 binding sites in rat brain membranes (Wong et al.,
1986). Electrophysiological studies on cultured neurones also demonstrated that MK801 selectively bound to and blocked open NMDA receptor channels (Huettner and Bean, 1987). A number of non-competitive antagonists were subsequently developed that interacted at the PCP binding site; one was tenocyclidine (TCP) which was derived from PCP and had over 10-fold higher affinity than PCP for the PCP binding site (Vignon et al., 1986). However, the most potent ligand discovered for the PCP binding site was MK801. This ligand had an affinity of 10 - 30 nM \textit{in vitro}, and was highly selective for the NMDA receptor (Wong et al., 1986; Huettner and Bean, 1988).

1.3.1.5 \textbf{The polyamine modulatory site}

The endogenous polyamines, spermine and spermidine, are found in high concentrations in the brain. However, their functions were largely unknown. They were first reported to increase the binding affinity of $[^3H]$MK801 to NMDA receptors \textit{in vitro} (Ransom and Stec, 1988; Williams et al., 1989). Electrophysiological studies showed that polyamines selectively enhanced NMDA-induced currents in some types of neuronal cells (Williams et al., 1990; Sprosen and Woodruff, 1990). However, spermidine did not displace $[^3H]$glycine or $[^3H]$CCP binding to the NMDA receptor (Ransom and Spec, 1988) suggesting they were interacting with a distinct polyamine modulatory site.

Polyamines were reported to have many modulatory effects, including, stimulation, increase in affinity for glycine, voltage-dependent inhibition and decrease in affinity for glutamate (McGurk et al., 1990; Williams et al., 1990, 1994). This led to their classification as agonists, partial agonists, antagonists and inverse agonists (Williams et al., 1990; Reynolds and Miller, 1989a). For example, spermine and spermidine simultaneously potentiated and inhibited responses at NMDA receptors (Benveniste and Mayer, 1993; Rock and MacDonald, 1992c). The inhibitory effects were voltage-dependent, possibly involving a modulatory site within the pore of the NMDA receptor channel
Figure 1.3  Schematic representation of the ligand binding sites of the NMDA receptor and channel

The NMDA receptor allows the passage of Na\(^+\) and Ca\(^{2+}\) into the cell, and K\(^+\) out of the cell. Glutamate (glu) and glycine (gly) are co-agonists of receptor function. Polyamines (PA) are positive modulators at low concentrations (PA 1 site), but may inhibit receptor function at higher concentrations by binding within the ion channel (PA 2 site). Mg\(^{2+}\) blocks the channel in a voltage-dependent manner.
CHAPTER 1

(Rock and MacDonald, 1992b). Polyamines that did not produce a potentiation response to NMDA, did show similar inhibitory effects to those produced by spermine and spermidine. This indicated the presence of two distinct sites for polyamine action on the NMDA receptor (Rock and MacDonald, 1992c; Donevan et al., 1992). This was supported by the pharmacology of polyamine site antagonists, such as ifenprodil, which is an atypical noncompetitive antagonist of the NMDA receptor (Reynolds and Miller, 1989b; Legendre and Westbrook, 1991).

In some cases the potentiation of NMDA receptor activity by low concentrations (i.e. less than 1 mM of spermine and spermidine) showed 'glycine-sensitive' and 'glycine-insensitive' components of action. This has been demonstrated in hippocampal neurones (Benveniste and Mayer, 1993). The glycine-sensitive component of potentiation was due to an increase in glycine affinity by the NMDA receptor. This resulted from a reduction of the rate of dissociation of glycine from the NMDA receptor (Benveniste and Mayer, 1993). In a subset of the hippocampal neurones a rapid initial component of potentiation was seen, due to polyamine evoked activity in NMDA receptors which had already bound glycine. This was the glycine-insensitive component of polyamine potentiation of NMDA receptors (Benveniste and Mayer, 1993).

1.3.2 Pharmacological Evidence for Regional and Developmental Heterogeneity of Native NMDA Receptors

Much evidence has been accumulated showing the existence of distinct subtypes of the NMDA receptor. The pharmacological evidence from electrophysiology and radioligand binding studies has been supported by advances in the molecular biology of the NMDA receptors (discussed in 1.3.3). The original evidence for NMDA receptor heterogeneity was from electrophysiological studies based on the regional variation in the CNS of the potency of quinololate, an NMDA receptor agonist (Perkins and Stone, 1983).
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Radioligand binding studies have also demonstrated NMDA receptor heterogeneity in adult rat brain. The pharmacological subtypes of the NMDA receptor were broadly located in the cerebellum, midline thalamic nuclei and forebrain (Vignon et al., 1986; Monaghan et al., 1988; Ebert et al., 1991; Monaghan and Beaton, 1992). Cerebellar NMDA receptors generally bound competitive antagonists with lower affinity than forebrain (Yoneda and Ogita, 1991). The pharmacological profile of the non-competitive channel blocking antagonists for cerebellar NMDA receptors also differed from that found for forebrain NMDA receptors (Ebert et al., 1991; Beaton et al., 1992). Furthermore, cerebellar [3H]MK801 binding appeared to be less sensitive to modulation by polyamines and Mg²⁺ than forebrain NMDA receptor [3H]MK801 binding (Yoneda and Ogita, 1991; Reynolds and Palmer, 1991). There were also suggestions that the glycine binding sites of forebrain and cerebellar NMDA receptors may have different pharmacological profiles (O' Shea et al., 1991).

NMDA receptors from the midline thalamic region and other midbrain regions were discovered to be similar to cerebellar NMDA receptors in their quinolinate and CPP pharmacology (Beaton et al., 1992). However, the pharmacology for the competitive antagonists, such as 6-tetrazole-decahydroisoquinoline-3-carboxylic acid (LY233536) had a higher affinity for the NMDA receptors of the mid-thalamic region. The non-competitive channel blocking antagonists such as MK801 also displayed a pharmacological profile distinct from cerebellar and forebrain NMDA receptors (Beaton et al., 1992).

Most NMDA receptors in the forebrain region displayed similar pharmacological profiles for agonists and antagonists. However, the affinities of compounds in different forebrain regions could be distinguished in quantitative autoradiography studies (Sakurai et al., 1993; Porter and Greenamyre, 1995). In the lateral thalamic nuclei, NMDA receptors displayed a higher affinity for antagonists than did receptors in regions such as medial striatum. Conversely, medial striatum NMDA receptors displayed a higher affinity for agonists than did
NMDA receptors in the lateral thalamus. These two populations have been termed the 'agonist' and 'antagonist' preferring regions (Monaghan et al., 1988; Sakurai et al., 1993).

In addition to NMDA receptor heterogeneity in adult mammalian brain, evidence of distinct NMDA receptor subtypes being expressed during early development was found. Binding studies showed that the polyamine site of the NMDA receptor was developmentally regulated. Williams et al. (1993) showed neonatal NMDA receptors had increased sensitivity to ifenprodil, due to the delayed appearance of a lower affinity binding component and decreased sensitivity to spermine. Moreover, electrophysiological studies suggested that neonatal NMDA receptors had a reduced sensitivity to Mg\(^{2+}\) (Ben-Ari et al., 1988). Expression of hippocampal mRNA from rats of different ages in *Xenopus* oocytes showed that glycine and Mg\(^{2+}\), but not NMDA potency varied with development (Kleckner and Dingledine, 1991). Hippocampal mRNA from 1-2 day old rats generated NMDA receptors which had a higher sensitivity to glycine, as compared to the hippocampal mRNA from 14-15 day old rats (Kleckner and Dingledine, 1991).

1.3.3 Molecular Biology of the NMDA Receptors

1.3.3.1 Cloning of the NMDA receptor

Due to the importance of NMDA receptors in brain physiology (discussed in 1.4 and 1.5) the cloning of the NMDA receptor subunit cDNAs was an eagerly awaited event. A partial protein sequence could not be obtained due to the difficulties in the solubilization and purification of the NMDA receptors. Therefore other techniques were utilized. Nakinishi's group isolated the first NMDA receptor subunit cDNA, utilizing cloning with functional expression in *Xenopus* oocytes (Moriyoshi et al., 1991). The clone, from a rat forebrain library, encoded for an NR1 subunit. The NR1 gene contained 22 exons and spread over
approximately 2.5 kb (Hollmann et al., 1993). Subsequent work by a number of other groups isolated further NMDA receptor subunit genes and splice variants, by screening brain cDNA libraries using the original clone as a probe (e.g. Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). Five NMDA receptor genes, NR1 and NR2A-D, have now been cloned for both rat and mouse, as well as several of the human homologues (e.g. Karp et al., 1993; Le Bourdelles et al., 1994).

The nucleotide sequence of NR1 showed a large open-reading frame of 2.8 kb, encoding a polypeptide of 938 amino acids in length, including a signal peptide of 18 residues at the N-terminal. The calculated relative molecular mass was 105500. A sequence comparison with the GluR1 subunit (discussed in 1.3.5) showed an overall homology of 22-26%. Multiple potential N-glycosylation and phosphorylation sites were identified in the NR1 polypeptide sequence. Hydropathy analysis predicted four hydrophobic segments (M1-M4) (Moriyoshi et al., 1991). These appeared to correspond to the 4 transmembrane segments seen in ligand-gated ion channels such as the nicotinic acetylcholine receptor (nAChR) and the γ-aminobutyric acid_A receptor (GABA_AR) (Betz, 1990) This topological model is shown in Figure 1.4 (model 1). However, further studies suggested that the initially proposed four transmembrane model (Moriyoshi et al., 1991) was incorrect. For example, Tingly et al. (1993) showed that the C-terminal region of the NR1 subunit was phosphorylated in vivo, implying that the C-terminal is intracellular. The intracellular location of the NR1 C-terminus was also demonstrated by the immunological studies of Chazot et al., (1995). Furthermore, mutational analysis of both the glycine-binding site and redox modulatory site of the NR1 subunit suggested an extracellular location for the region between M3 and M4 (Kuryatov et al., 1994; Sullivan et al., 1994; see 1.3.3.5). These observations could be explained by a three transmembrane model, with a pin-loop segment forming the M2 region, as shown in Figure 1.4 (model 2). Studies on non-NMDA subunit topology (see 1.3.5) also support this model.
Figure 1.4  Representations of the topological models suggested for the ionotropic glutamate receptor subunits

Model 1, the 4-transmembrane domain model, which was originally proposed. It was based on hydropathy profiles and assumed homology with other ligand-gated ion channels and is now thought to be incorrect. Model 2, the 3-transmembrane model with a pin-loop M2 region. This is the currently proposed model, see 1.3.3.1 and 1.3.5.1. Y represents potential glycosylation sites. P represents potential phosphorylation sites
The first cDNA of the NR2 genes, NR2A (also termed NRε1), was isolated from a mouse forebrain cDNA library by screening with mouse AMPA/kainate cDNA probes (Meguro et al., 1992). This was followed by the cloning of three other genes NR2B, NR2C and NR2D by screening and polymerase chain reaction (PCR) amplification (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993). The NR2A-2D subunits shared ~40-50% amino acid homology, but had only ~15% amino acid identity with the NR1 subunit. A notable feature of the NR2 subunits was the extended C-terminus of approximately 600 amino acids for the NR2A and NR2B subunits, and approximately 400 amino acids for the NR2C and NR2D subunits. The predicted mature subunits for mouse NR2A, NR2B, NR2C and NR2D were composed of 1445, 1456, 1220 and 1296 residues with molecular masses 163267, 162875, 133513 and 140656 respectively. As with the NR1 subunit, hydrophobicity analysis predicted 4 membrane regions (M1-M4). They are located in the polypeptide as shown in Figure 1.5. As in the NR1 subunit an asparagine (N) residue occupies the important position for Ca\textsuperscript{2+} permeability in the putative channel-forming, M2, region. The topology of the NR2 subunits is assumed to be the same as the NR1 subunits and the non-NMDA receptor subunits.

The cloning of the mouse form of NR1, also known as NRζ1, suggested the existence of two forms of the subunit (Yamazaki et al., 1992). Other laboratories subsequently described the existence of eight isoforms of the NR1 subunit (Sugihara et al., 1992; Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Durand et al., 1993; Hollmann et al., 1993). The isoforms are schematically shown in Figure 1.6. These isoforms were generated by alternative splicing of the NR1 gene, with the insertion or deletion of three exons. Isoforms with deletion II (NR1d, NR1e, NR1g and NR1h) generated a different C-terminus as compared to the originally cloned NR1 subunit (NR1a). The NR1 subunit splice variants are summarized in Figure 1.6. The mature NR1
Figure 1.5  A comparison of the polypeptide sequences encoded by the mouse NR1a, NR2A and NR2C cDNAs

Identical amino acids are represented by (*), conservative substitutions are represented by (.). The putative membrane regions, M1-M4, are indicated by (—). The signal peptide is shown. Numbering of the sequence starts from the first amino acid of the mature peptide. The sequence alignment was performed by PC/gene software package, using the method of Higgins and Sharpe (1988).
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leading peptide - 1
RAAC-----DPKVVTGAVLSTRKHEQMREAV 28

NR1a MSTM---HILFITALFSQSFSA 1
NR2a MG-LASYQLVLEAL---VWNGPA 2
NR2c MLGALC---PALLLSGLAGA 4

NR2c TTRL-TQNFPLDPEIQFPTIGWNVTPSFLSILQTCGLLGAARBVHGIYFED-+V 80

NR1a NOQANKRHGWSKIQINATSRT---HKPNAIQONLVSCEOILSSQAVYAILVSHPTP 80

NR2a RNLWGEQQTGQLPLDNNVLWALLMNRPDKSLITVENCDLMGSARHFLVFGD-+ 82

NR2c TRL-TQNFPLDPEIQFPTIGWNVTPSFLSILQTCGLLGAARBVHGIYFED-+V 80

NR1a NDHFPTPVPSTAGFYRIPVLGGLTTRMS---IYSDKSIHLSFLRTVPPPSYQCSVWVF 134

NR2a DQEAQMLDPSSTQFTPILGLIHGGASMIMADKFTPTTFPQFAGASIQQATVMKL 137

NR2c DQEAQMLDPSSTQFTPILGLIHGGASMIMADKFTPTTFPQFAGASIQQATVMKL 137

NR1a ALYAL--PDIIGLQILGLVESASHDASYGVAARVHLEK-+ENTIDPFPRGC 290

NR2a ELIPKEFPSGLISVSYDDWDYSLEARVRDGILGILTTAASSMLEKSFYIEPKASC 298

NR2c ELIPKEFPSGLISVSYDDWDYSLEARVRDGILGILTTAASSMLEKSFYIEPKASC 298

NR1a ALYAL--PDIIGLQILGLVESASHDASYGVAARVHLEK-+ENTIDPFPRGC 290

NR2a ELIPKEFPSGLISVSYDDWDYSLEARVRDGILGILTTAASSMLEKSFYIEPKASC 298

NR2c ELIPKEFPSGLISVSYDDWDYSLEARVRDGILGILTTAASSMLEKSFYIEPKASC 298

NR1a VQVGIYNGTHVIPNDRKIIWPGGETEKPRGYQMSTRLKIVTHIQEPFVYVK-PTM 397

NR2a EKVGKWE---NQTLRLHAWPRYKSFSDCEPD-DNLHSLVTTEAEEPFIVZERO 402

NR2c EKVGKWE---NQTLRLHAWPRYKSFSDCEPD-DNLHSLVTTEAEEPFIVZERO 402

NR1a SDTGKEEFYNPGQPKKVICTGPTPDGPRTEVQCCYCGFVCVDDILKCLMRT 452

NR2a LTETC-----VPNVTFCRRKVF---KIN-NSTNEQMNVRKCCGGFCICDILKLSSRTV 449

NR2c LGTGCC-----VPNVTFCRRKVF---KIN-NSTNEQMNVRKCCGGFCICDILKLSSRTV 449

NR1a NFYEVHVLADKFGQFERVNNSSKKEWNGSVKMGGAQLLVQAMVGLTINNEAO 507

NR2a KFYTALVLVTVNGHKGK--KVNVT--WNGMIQGEEYYVRQAVMAGSGLTINNEERSE 498

NR2c KFSYALVLVTVNGHKGK--RVRGV---WNGMIQGEEYYVRQAAMIGSGLTINNEERSE 499

NR1a YIEFSKPFYQKQLTFLKKEIPRSTLSDSMQPFQSTLLWLV-GLSVHVAVMVL 561

NR2a VVSFVVFVFETIGSWVMRSNVTSPSAFALFESSASMVFMMLLIVSAIAQFV 553

NR2c IISFKVFVFSGWNSNVTSPSAFALFESSASMVFMMLLIVSAIAQFV 554

NR1a DLFSP-----FGRFKVNESEEEEDEALTLSSWMSWSWVLNLSSGIGEAGPRSFSTAL 614

NR2a FYEFPSVYNNLAKGKAHPHSTIGKAIWLLGLVNFNSVPQONPKGTSKIM 608

NR2c FYEFPSVYNNLAKGKAHPHSTIGKAIWLLGLVNFNSVPQONPKGTSKIM 608

NR1a GMWAGFMIIIVASYANLALAFILLVLDRPEERITGINQDPLNPIPSD---FQTV 666

NR2a VSWAFAVFLFASYANLALAEMIEZEFVQTVGLSDKKQRFHDYSPFRFGTV 663

NR2c VSWAFAVFLFASYANLALAEMIEZEFVQTVGLSDKKQRFHDYSPFRFGTV 663

NR1a GMWAGFMIIIVASYANLALAFILLVLDRPEERITGINQDPLNPIPSD---FQTV 666

NR2a VSWAFAVFLFASYANLALAEMIEZEFVQTVGLSDKKQRFHDYSPFRFGTV 663

NR2c VSWAFAVFLFASYANLALAEMIEZEFVQTVGLSDKKQRFHDYSPFRFGTV 663
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NR1a  KQSSVDIYFRQQVELSTMYRHEKHYESAIAQAVRDNLHAFIWDSAVLEF  721
NR2a  PNGSTERNIRN—NYPHYOMYMTFQNGVEDALVSLKTGDFAIFYDAAVLYNK  716
NR2c  PNGSTERNIRN—NRDMHITMVFKNQSUVELTLSKMGFLDAIFYDAAVLYNM  717

NR1a  ASQK--CDLVTIGF--LFFRSFQGGLGMKDSFKDSFQWQNYLSILKSHENGFMEDLDK  772
NR2a  AGQDEGKVLTGSGYIFATTGIGALQGKSEFQRDFDLALLQFGDGEMEELET  771
NR2c  AGQDEGKVLTGSGVFSAGTVGIGVAMQGDSHWRKRAIDLALQFGDGQETQLET  772

M4

NR1a  TWWRYQECDSRNGA—PAITLFEMAGVLVAGGIVAGIFLFI----------  815
NR2a  LWLT-GICHNEKNEVMSQIDLNNAGVFYMLAANMLSLLIFPWEHLFWYKLR  825
NR2c  VWLS-GICHNEKNEVMSQIDLNNAGVFYMLAANMLSLLIFPWEHLFWYKLRH  826

NR1a  EAYKR-------------HKDARRKQMQLAFANWV----------  838
NR2a  CPTGCVSDRFLPSNVRHISGIGCHVGHVI---EEKKSPFDNLTSQSNMLKLR  877
NR2c  SVPS--SSQDFFLLAFSRGITYCENGFQVSLFSRPSDPLTAGTAQANVLMKLQ  879

NR1a  AARMDVASVDSG-S-LRDRATIE--NGWNNRRAPAPTTSGPRCTPGPGQSP  931
NR2a  -VWRKLRQQDK-------------SGRAEDP  856
NR2c  SGWRPPGGTAGFRLRAAPQFQPRQAPQGAPQGRLSFTCPDEHAPLGMFL-  985

NR1a  KKKATRAITSLA-------------SSFRK---------  875
NR2a  RPPQWKKSELSRNLQSLNQN---PVQSDRKTAEATRTHSLKSPRYP---  1031
NR2c  RORTSREPARRALPERSLHLHHAYSSFFPRAERSGPRFILFPPFPEPPFDLPLL  1040

NR1a  RRSXEDTSTGGGRGALQOKQDOQTDLPRRAIE--  905
NR2a  -EEEVHDJSTERSSRATCREPDSDKNNKHKTNKRNKRMSKYPDCSEVETYVK  1085
NR2c  PEQLMREALLRAAWARPQRPHASLPSVSAEAFTSR---NLPRARCTGHACAC--  1091

NR1a  ---------------REE  908
NR2a  TKASSSRDKTYTIDEGKPSHDLFDQQIFENIVLPENPFDITYQDMNENFKDG  1140
NR2c  ---PCQPSRSCRHRVAQTFQLSRIL---PSYREACVEGVPAGVAATWQPGVRCHLTH  1141

NR1a  GQIQLCS-------------RHRRES  920
NR2a  STLPMRNRPLNHEGDLNDQNYKLYAKHTLKDKGSPH--SEGSDRURYQNSTHR  1193
NR2c  THLPFCWTCVRHFPSCSSHPWHLGHTWEPSPHRGRTGLGTGSRDSVLEEVS  1196

NR2a  SCLSNLPTSGHFTMRSFPPCDACLKMGNYLIDDLEQMLQETGPNPATREEAYQOD  1248
NR2c  EACGTQGFPSCTWRRSSLESEV  1223

NR2a  WSQNNALQFKVNLKINQRYSHYMDNLKPREIDLSRPSRSISLKDRELELGICY  1303
NR2a  GSLFSVPSSSKLGNKSSLPSQGLEDKRSKSKSLKHPSNDPFLNYTDQGQLVIG  1358
NR2a  RCPSDYPKHLPSQAVNMDYSLSRLSSTASYACRDRSGHSDVYI5EHMYAANK  1413
NR2a  NNMYSTPRVINCSNRVYKMPIESDV  1442
Figure 1.6  Schematic representation of the NR1 splice variants and the NR2 subunits

The NR1 splice variants are called by the nomenclature of Sugihara et al. (1992). The nomenclature in brackets is that of Hollmann et al. (1993).
splice variants ranged in size from 883-941 residues, of molecular masses 97.3-105.9 kDa. The nomenclature used for the NR1 splice variants, as shown in Figure 1.6 is based on Sugihara et al. (1992), the nomenclature in brackets is from Hollmann et al. (1993).

The interspecies homology of the NMDA receptor subunits was high. The rat and mouse homologues of NR1a showed over 99% amino acid sequence identity, with only two, conservative amino acid substitutions of E195 to N and I442 to V in the mouse subunit. The human NR1 subunits also showed high sequence conservation, compared to the rat NR1, with 90% nucleotide sequence homology and only 4 amino acid substitutions (Karp et al., 1993). The NR2 subunits were found to be more diverse between species. The mouse and rat NR2C polypeptides showed 95% homology (Ishii et al., 1993). The human NR2A subunit has 81 amino acid changes from the rat sequence, many of which occurred in the C-terminal part of the polypeptide (Le Bourdelles et al., 1994).

1.3.3.2 Regional and developmental distribution of NMDA receptor subunit mRNAs

Distribution of the NMDA receptor subunit mRNA in adult rat brain was analysed by in situ hybridization. The NR1 subunit mRNA was found to be widely distributed throughout the brain and expressed in nearly all neuronal cells (Moriyoshi et al., 1991). In contrast, the NR2 subunits showed a well-defined expression pattern (Ishii et al., 1993). The most widely expressed subunit was NR2A, with in situ labelling seen in the cerebral cortex, hippocampus, caudate-putamen, thalamus and olfactory bulb. The NR2B subunit was expressed only in the forebrain. In contrast NR2C mRNA was prominently detected only in the cerebellum. Low levels of the NR2D subunit mRNA were found in the thalamus, brainstem and olfactory bulb (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993).

Studies showed that expression of the NR2 subunit mRNAs were
developmentally regulated. In embryonic brain only NR2B and NR2D were seen; NR2B widely, while NR2D is restricted to the diencephalon and brainstem. Over postnatal days 1-14 the NR2A mRNA appeared in the entire brain and the NR2C mRNA in the cerebellum. The expression of NR2B became restricted to the forebrain and the level of NR2D was greatly reduced (Akazawa et al., 1994; Monyer et al., 1994; Watanabe et al., 1992).

The NR1 splice variant mRNAs also showed some regional specificity. The N1 exon containing splice variant mRNAs (NR1b, NR1f, NR1g and NR1h) were most prominently seen in the hippocampal CA3 region, thalamic nuclei and cerebellar granule cell layers (Standaert et al., 1994). Probes which labelled N1 exon-lacking splice variants (NR1a, NR1c, NR1d and NR1f) gave prominent labelling throughout the CA1, CA2, CA3 and dentate gyrus of the hippocampus, the striatum and granule cell layer of the cerebellum. Probes which detected both NR1a and NR1b showed labelling in the hippocampus, cortex and caudate. A probe for NR1e and NR1g showed prominent labelling in the thalamus, cerebellum and hippocampus (Laurie and Seeburg, 1994b).

1.3.3.3 Immunocytochemical localization of NMDA receptor subunits

Many investigations have studied the mRNA distribution of the NMDA subunits. However, the presence of mRNA does not always imply the presence of the protein, for example NR1 mRNA is abundantly expressed in the PC12 cell line, however, only trace amounts of protein were detected and no functional NMDA receptor found by electrophysiology (Sucher et al., 1993). Immunocytochemical studies allow the confirmation of the presence of the polypeptide and possibly its ultrastructural localization within the neurone.

Brose et al. (1993) used a polyclonal NR1 antibody raised against a peptide sequence from the protein loop between the putative membrane regions M3 and M4. This antibody demonstrated the presence of the NR1 protein throughout the rat brain, agreeing with the in situ hybridization studies (see
The NR1 polypeptide was most prominent in layers 2, 3 and 5 of the cerebral cortex, the hippocampus and the cerebellum. Studies by Petralia et al. (1994b) used a polyclonal antibody against the C-terminal region that recognized 4 of the NR1 splice variants, NR1a, NR1b, NR1c and NR1f, and also showed widespread labelling of the rat brain sections. The most densely stained cells included the Purkinje cells of the cerebellum and the pyramidal and hilar neurones of the CA3 region of the hippocampus, less dense staining was seen in the neocortex, striatum and olfactory bulb. Ultrastructural localization of the immunocytochemical staining in the hippocampus, cerebral cortex and cerebellar cortex was examined. The major staining was seen in the post-synaptic densities and associated dendrites. The pre-synaptic terminals were unstained (Petralia et al., 1994b). A study by Bilak et al. (1995) with a monoclonal NR1 antibody in the cerebellar cortex supported the results of Petralia et al. (1994b). Heavy staining was seen in Purkinje cell bodies and proximal dendrites and granule cell bodies and dendrites, while little or no staining was seen in the stellate and basket cells, Bergmann's glia and golgi type II cells (Bilak et al., 1995).

Studies with a polyclonal antibody which detected both NR2A and NR2B, showed significant staining throughout the nervous system, including the olfactory bulb, cerebral cortex, hippocampus and caudate-putamen. The most intense staining was seen in the cortex and hippocampus, with the hindbrain and cerebellum more weakly labelled (Petralia et al., 1994a). The ultrastructural localization of the NR2 subunits was similar to that seen for the NR1 subunit, staining was most prominent in the dendrites and post-synaptic membranes and densities (Petralia et al., 1994a). Studies have also been carried out with NR2C subunit-specific antibody (Mulac-Jericevic et al., 1993). This antibody strongly labelled cerebellar granule neurones in both the inner and outer molecular layer of the cerebellum, but not hippocampal neurones. These results match the labelling seen with in situ hybridization studies (see 1.3.3.2).
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1.3.3.4 Biochemical characterization of native NMDA receptors

Sheng et al. (1994) demonstrated the co-association of NR1 and NR2 subunits in native membranes by immunoprecipitation. Antibodies against NR1 subunits co-precipitated both NR2A and NR2B subunits from rat cortex. Antibodies against NR2A or NR2B subunits also immunoprecipitated the NR1 subunits, however, they also co-precipitated NR2B and NR2A subunits respectively. This demonstrated the association of NR2A and NR2B subunits in the same complex in vivo. Functional NMDA receptors are not formed in the absence of the NR1 subunits (e.g. Monyer et al., 1992). Therefore, this suggested a proportion of the native cortical NMDA receptors are hetero-oligomers of three subunit types, NR1, NR2A and NR2B (Sheng et al., 1995).

Attempts to determine the molecular size of the NMDA receptor complex have been hampered by the difficulties of purifying the NMDA receptor. Brose et al. (1993) employed two independent techniques, chemical-cross linking of proteins in synaptic plasma membranes and size-exclusion chromatography of synaptic membranes, solubilized in Triton X-100 and performed in the presence of 1% sodium cholate. The presence of the NMDA receptor complex was detected using an antibody against the NR1 subunit. The initial procedure gave a molecular mass of 730 kDa, whilst the second technique suggested a size of 630 kDa for the NMDA receptor complex. However, degradation of the NR1 protein occurred during the chromatographic procedure and the size resolution of this procedure was inherently lower than the cross-linking technique. Therefore, the 730 kDa was considered the more accurate estimation of the NMDA receptor complex size. Studies on non-NMDA receptors suggested a pentameric stoichiometry (Blackstone et al., 1992; Wenthold et al., 1992; see 1.3.5.4). Moreover, other ligand-gated ion channels such as the nAChR receptor have a pentameric structure. The 730 kDa size estimate would agree with the presumed pentameric structure of the NMDA receptor, with the suggested stoichiometry of 2 NR1 subunits and 3 NR2 subunits (Behe et al., 1995).
1.3.3.5 Properties of recombinant NMDA receptors

The expression of NMDA subunit clones in various expression systems has been used to elucidate the properties and functions of the individual subunits within the NMDA receptor. These studies are discussed in more detail below.

Homomeric receptors

The original cloning of the NR1 subunit cDNA followed by its expression in *Xenopus* oocytes showed that it formed a functional homomeric receptor-channel complex. This homo-oligomer had the pharmacological properties of the native NMDA receptor, which included, activation by L-glutamate, modulation by glycine, Ca\(^{2+}\) permeability, Zn\(^{2+}\) inhibition and a voltage-dependent block by Mg\(^{2+}\) (Moriyoshi *et al.*, 1991). Application of NMDA (100 μM) to NR1 mRNA injected *Xenopus* oocytes elicited an inward current of 40-70 nA, under voltage clamp at -80 mV in a Mg\(^{2+}\)-free medium. The response to NMDA was abolished by application of Mg\(^{2+}\) (100 μM). The Mg\(^{2+}\) block was absent at holding potentials more positive than -20 mV, thus demonstrating voltage-dependent block. A requirement for glycine was also demonstrated (Moriyoshi *et al.*, 1991). The actions of agonists and antagonists selective for NMDA receptors were also studied. The agonists L-glutamate, ibotenate, quisqualate and homocysteate all elicited a response, with L-glutamate being the most potent. Competitive antagonists, such as AP5 and CPP reduced the NMDA response, as did the non-competitive antagonists, MK801 and 7-chlorokynureate (7-CK) (Moriyoshi *et al.*, 1991). Sensitivity to protein kinase C (PKC) activators and modulation by polyamines has also been demonstrated for homomeric NR1 receptors expressed in *Xenopus* oocytes (Yamazaki *et al.*, 1992).

Expression of NR1 alone in mammalian cell lines, following transient or stable transfection, did not form functional ion channels as determined by electrophysiology (Monyer *et al.*, 1992; Boeckman and Aizenman, 1994; Grimwood *et al.*, 1995). The evidence from \(^{[3]H}\)MK801 binding was more contradictory. Some reports showed homomeric expression of NR1 gave high
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affinity \(^{3}H\)MK801 binding (Chazot et al., 1992; Laurie and Seeburg, 1994a). Other studies observed no \(^{3}H\)MK801 binding following expression of NR1 in a mammalian cell line (Grimwood et al., 1995). However, the studies by Grimwood et al. (1995) demonstrated the formation of a high-affinity binding site for glycine site antagonists. Homomeric NR1 receptors have also been expressed in an insect cell line (Sf21 or Sf9) using a baculovirus vector (Kawamoto et al., 1995). Cell membranes from NR1 cDNA infected Sf cells demonstrated high levels of \(^{3}H\)DKA binding, but showed no specific binding with radiolabelled (E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid (\(^{3}H\)CGP-39653) or \(^{3}H\)MK801 (Kawamoto et al., 1995).

The NR1 isoforms were expressed in Xenopus oocytes as homomeric receptors to determine the effects of splice variants on receptor function. Studies were carried out on homomeric NR1 receptors differing by a single insertion or deletion. Investigation of antagonists of the NMDA receptor, such as AP5, CPP, MK801 and Mg\(^{2+}\) showed that while they all inhibited an NMDA response in homomeric NR1 receptors, there were no significant differences in effectiveness between the isoforms (Sugihara et al., 1992). It was shown that NR1 subunits lacking the N1 insert (NR1a, NR1c, NR1d and NR1e) have a higher affinity for agonists and potentiation by spermine at high glycine concentrations. In contrast subunits containing the N1 insert (NR1b, NR1f, NR1g and NR1h) exhibited threefold larger current amplitudes (Hollmann et al., 1993; Zheng et al., 1994). All the splice variants exhibited potentiation by spermine at non-saturating concentrations of glycine (Zhang et al., 1994). Isoforms which lacked the N1 insert showed potentiation by submicromolar concentrations of Zn\(^{2+}\). However, variants which have the N1 insert showed inhibition by high concentrations of Zn\(^{2+}\), but did not show any potentiation. This was also true of any NR1 splice variant co-expressed with NR2A (Hollmann et al., 1993; Zheng et al., 1994). The NR1 isoforms also varied in their sensitivity to activators of PKC. The highest potentiation (20-fold) by phorbol 12-myristate 13-acetate (PMA) (a PKC
activator) was shown by homomeric NR1\text{g} channels, whereas, homomeric NR1a channels showed only 3-fold potentiation. Further studies showed that the N1 and C2 cassettes were equally important in controlling sensitivity to PKC activators (Durand et al., 1992 and 1993). Tingley et al. (1993) also demonstrated that phosphorylation by PKC of NR1 subunits transiently expressed in HEK 293 cells was regulated by alternative splicing of the C-terminus. This provided the first evidence that the C-terminal domain of the NR1 protein may be located intracellularly (see 1.3.3.1).

**Heteromeric receptors**

Homomeric expression of the NR2 subunits in *Xenopus* oocytes did not give functional receptors (Monyer et al., 1992). However, co-expression of NR1 and NR2 subunits gave a large electrophysiological response to agonists, which was generally 10-100-fold greater than the response from homomeric NR1 receptors (Monyer et al., 1992). The exception to this was the NR2D subunit. Ikeda et al. (1992) reported only a 5-fold increase in current amplitude for NR1/NR2D receptors in *Xenopus* oocytes, while Ishii et al. (1993) reported no increase at all. The co-expression of NR1 with NR2A-D subunits gave receptors with different pharmacological profiles in agonist affinities, antagonist effectiveness and sensitivity to Mg\(^{2+}\) block (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Laurie and Seeburg 1994a). The heteromeric NR1/NR2D receptor had the highest affinity for L-glutamate and glycine, with EC\(_{50}\) values of 0.4 \(\mu\)M and 0.09 \(\mu\)M respectively. The order of affinities was NR1/NR2D > NR1/NR2C > NR1/NR2B > NR1/NR2A. The EC\(_{50}\) values of the NR1/NR2A receptor for L-glutamate and glycine being 1.7 \(\mu\)M and 2.1 \(\mu\)M respectively (Ikeda et al., 1992; Kutsuwada et al., 1992). The sensitivity to competitive antagonists also varied. The order of affinity for AP5 was NR1/NR2A > NR1/NR2B > NR1/NR2C > NR1/NR2D. However, for the glycine site competitive antagonist 7-CK, the order of sensitivity was NR1/NR2C > NR1/NR2B > NR1/NR2A ~ NR1/NR2D (Ikeda et
The NR1/NR2A and NR1/NR2B channels were more sensitive to Mg$^{2+}$, Zn$^{2+}$ and (+)MK801 than the NR1/NR2C and NR1/NR2D receptors (Ikeda et al., 1992; Kutsuwada et al., 1992). However, the sensitivities to PCP, ketamine and N-allylnormetazocine (SKF-10047) were only slightly variable between the NR1/NR2 heteromers (Yamakura et al., 1993). The effects of extracellular Ca$^{2+}$ on the heteromeric channels expressed in *Xenopus* oocytes were differential. The initial spike which was seen was due to Ca$^{2+}$-activated Cl$^{-}$ channels (Leonard and Kelso, 1990). The steady-state responses of NR1/NR2A, NR1/NR2C and NR1/NR2D were suppressed by extracellular Ca$^{2+}$, whereas the response of the NR1/NR2B channel was potentiated. This suggested differences in Ca$^{2+}$ sensitivity between the heteromeric NMDA channels. However, the Ca$^{2+}$ permeability of the four heteromeric channels is similar (Monyer et al., 1994). The observed differences in Ca$^{2+}$ sensitivity of the recombinant NMDA receptor channels may be due to the ability of Ca$^{2+}$ to act as a permeant blocker of the NMDA channel. The strength of the block depends upon the NMDA receptor subunit composition (Burnashev et al., 1995).

Unlike the homomeric NR1 receptors, functional heteromeric NR1/NR2 receptors have been successfully expressed in mammalian cell lines (Grimwood et al., 1996; Stern et al., 1994). McIlhinney et al. (1996) showed NR1 required an NR2 subunit for cell surface expression in a mammalian cell line. This was necessary for both transiently and stably transfected mammalian cell lines. The single channel properties of heteromeric NR1/NR2 receptors have been characterized in *Xenopus* oocytes and HEK 293 cells (Stern et al., 1992, 1994). The expression system did not influence the fundamental properties of the NR1/NR2 receptors. Both NR1/NR2A and NR1/NR2B had two conductance levels, 50 pS openings and 40 pS sublevels of similar mean life times and frequencies. The conductance levels and amplitude histograms for these receptors were similar to those reported for CA1 hippocampal pyramidal cells (Gibb and Colquhoun, 1992). The NR1/NR2C channel gave conductances of 36 pS and 19
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pS. These were similar to cultured large cerebellar neurons. In developing
cerebellum, pre-migratory and migrating granule cells express NMDA receptor
channels with 50 pS and 40 pS conductances. However, mature post-migratory
cells express 33 pS and 20 pS conductances, in addition to the 50 pS and 40 pS
conductances (Farrant et al., 1994). The 33 pS and 20 pS conductances were
similar to those reported for the NR1/NR2C channels (Stern et al., 1992).

1.3.3.6 Mutagenesis studies on recombinant NMDA receptors

Site-directed mutagenesis studies have been used to identify functionally
important sites of the NMDA receptor. Much work was carried out on the M2
regions of the NMDA subunits, as these are believed to line the channel pore of
the NMDA receptor. The ligand binding regions have also been studied,
especially the glycine binding site. The subunit stoichiometry of the NMDA
receptor has also been examined by mutagenesis. These studies are discussed in
more detail below.

The channel pore region

It was shown that the arginine at position 598 (in the putative M2 region)
in the GluR2 subunit determined the Ca\(^{2+}\) permeability of the AMPA receptors
(Burnashev et al., 1992a). The equivalent position in the M2 region of the
NMDA subunits is occupied by an asparagine. Mori et al. (1992) carried out
point mutations on this site to determine its importance in Ca\(^{2+}\) permeability and
Mg\(^{2+}\) blockade. When the asparagine was replaced by a glutamine (N598Q) in
the NR1 subunit, heteromorphic receptors NR1(N598Q)/NR2B were less sensitive
to Mg\(^{2+}\) block. The corresponding mutation in NR2B (N589Q) also reduced the
sensitivity of the heteromeric channel, NR1/NR2B(N589Q) to blockade by Mg\(^{2+}\).
Burnashev et al. (1992b) also demonstrated this with the corresponding NR2C
mutation N593Q. Mutation of the NR1 asparagine to arginine (N598R) and co-
expression of NR1(N598R)/NR2A in Xenopus oocytes completely abolished
Mg\(^{2+}\) block even under highly hyperpolarized conditions. The Ca\(^{2+}\) permeability
of the NR1(N598R)/NR2A channel was also reduced (Sakurada et al., 1993). The NR1(N598Q) mutation reduced the sensitivity of NR1/NR2A channels to MK801 block and NR1(N598R) mutation abolished MK801 blockade. This pattern was seen for other channel blockers; PCP, ketamine and SKF-10047. The N to Q mutation reduced the sensitivity of the NR1/NR2A channels to blockade by varying extents, while the N to R mutation abolished sensitivity (Yamakura et al., 1993). These mutations also reduced sensitivity to 10 μM Zn$^{2+}$, although the mutant channels were still strongly inhibited by 100 μM Zn$^{2+}$ (Mori et al., 1992; Sakurada et al., 1993). These studies suggested that Mg$^{2+}$ and MK801 interacted at a common site within the NMDA receptor pore. Mutation of the five charged glutamic acid residues on the N-terminal side of the NR1 M2 region to the GluR1 equivalents had no effect on Mg$^{2+}$, MK801 or Zn$^{2+}$ inhibition, nor did individually changing them to glutamine (Mori et al., 1992; Sakurada et al., 1993).

**Ligand binding regions**

Studies by Wafford et al. (1995) on the human NR1a subunit identified two residues important in determining glycine affinity. The residues, aspartic acid 463 and lysine 465 (located on the N-terminal side of M1) were conservatively mutated to asparagine and glutamine respectively and co-expressed with NR2A in *Xenopus* oocytes. The NR1a(K465Q)/NR2A receptor had a 126-fold reduced affinity for glycine, while the D463N mutation reduced glycine affinity by 7-fold. Importantly the affinity for glutamate of the mutant receptors were only slightly lower than for the wild-type receptors. Mutation of the D481 residue to alanine or lysine further reduced glycine affinity by 100-fold and 150-fold respectively. Glycine site agonists also showed reduced affinity for both mutant receptors, although less so than glycine. However, the efficacy of the agonists, as compared to glycine, was unaffected. Antagonists of the glycine site, L-689560 and L-687414 (a derivative of HA-966) also showed reduced affinity for the D463N and K483Q mutants (Wafford et al., 1995). These residues had been
shown to play a role in glycine affinity by Kuryatov et al. (1994). A domain between the M3 and M4 regions of the NR1 subunit which contributed to glycine binding was also identified by Kuryatov et al. (1994). Mutation of valine 666 and serine 669 to alanine and glycine respectively, increased the EC$_{50}$ values for glycine by 13-fold and 25-fold respectively. The glutamate dose-response was not significantly altered by these mutations. Interestingly, the IC$_{50}$ values for the glycine antagonist 7-CK were not affected. The residues glutamine 387, phenylalanine 390 and tyrosine 392 in the N-terminal region were also important in determining glycine affinity. It was found that aromatic residues at 390 and 392 were necessary to maintain glycine affinity. However, if these residues were substituted to alanine, this did not significantly affect glutamate affinity. Replacement of glutamine 387 for lysine reduced the glycine EC$_{50}$ value by over 10000-fold, while the glutamate affinity was reduced by only 13-fold. Substitution of these residues markedly reduced inhibition by 7-CK (Kuryatov et al., 1994).

Laube et al. (1993) demonstrated that the cysteine residues, 402 and 418 in the NR1 subunit were crucial for glutamate-glycine interaction, although substitution for alanine did not significantly alter the NR1/NR2B channel affinity for glutamate or glycine. This suggested C402 and C418 formed a disulphide bridge essential for conformational changes with agonist binding.

A comparison of the NR1 subunit with bacterial periplasmic amino acid binding proteins revealed that the positions identified as important for glycine binding (by mutagenesis) did corresponded to bacterial protein residues known to interact with bound ligand. The 3-dimensional structure of the lysine/arginine/orthinine-binding protein (LAOBP) has been determined (Kang et al., 1991; Oh et al., 1993) and it is similar to the 3-dimensional structures of other bacterial periplasmic binding proteins despite low primary structure homology. These known structures could provide a basis for the modelling of the glycine-binding site of the NR1 subunit (Kuryatov et al., 1994).
Stoichiometry of recombinant NMDA receptors

Mutagenesis studies have also been used to investigate NMDA receptor stoichiometry. Behe et al. (1995) coexpressed wild-type NR1, mutated NR1(N598R) and wild-type NR2A in Xenopus oocytes and recorded single-channel conductances. The presence of the N598R mutation in the NR1 subunit substantially reduced single channel conductance of NR1/NR2A receptor channels. Analysis of the measured single channel amplitudes implied the presence of two NR1 subunits in the recombinant NMDA receptor. This was consistent with the suggestion of two binding sites for glycine and glutamate per NMDA receptor, which also implied the presence of two NR1 subunits per NMDA receptor (Benveniste and Mayer, 1991).

1.3.4 Molecular Pharmacology of the non-NMDA Receptors

As described earlier (see 1.3), pharmacological studies in the late 1960s suggested the existence of multiple types of glutamate receptor. The isolation of kainic acid from Digenia simplex, was followed by the discovery of quisqualate from the plant Quisqualis. Electrophysiological studies in the late 1970s demonstrated that these two compounds, together with NMDA (see 1.3.1) were agonists at different types of glutamate receptor. These receptors were initially named after these agonists. However, it was later found that quisqualate could activate the mGluRs (see 1.3.6), Therefore, the quisqualate receptors were renamed AMPA receptors after the more selective AMPA agonist (Watkins et al., 1990). However, as there were no antagonists that could clearly distinguish AMPA and kainate receptors, they were often referred to as the non-NMDA receptors (reviewed in Bettler and Mulle, 1995). A lack of specific antagonists has hampered the functional discrimination of AMPA and kainate receptors in vivo, although the classical non-NMDA receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 6-cyano-7-nitroquinoxaline-2,3-dione
(CNQX) showed some selectivity for AMPA receptors (Honoré et al., 1988). However, newly developed compounds are reported to have a greater selectivity; NS-102 reversibly blocked kainate receptor responses, and only minimally affected AMPA receptor responses to kainate (Johansen et al., 1993; Lerma et al., 1993). The compound 1-(4-aminophenyl)-4-methyl-7,8-methyl-enedioxy-5H-2,3-benzodiazepine (GYKI 52466) has been reported to be a highly selective, non-competitive AMPA receptor antagonist (Donevan and Rogawski, 1993). Previously the order of efficacy for the classical agonists was used to distinguish kainate and AMPA receptors. AMPA receptors showed a rank order of potency of AMPA ~ domoate > L-glutamate > kainate, while kainate receptors had a rank order of potency of domoate > kainate > L-glutamate >> AMPA. Moreover, the desensitization properties of the non-NMDA receptors to these agonists allowed the differentiation of the AMPA and kainate receptor subtypes (Bettler and Mulle, 1995). The benzothiadiazides, for example, cyclothiazide, selectively potentiated and inhibited the desensitization of AMPA receptors but they were inactive at kainate receptors (Partin et al., 1993; Wong and Mayer, 1993).

Autoradiographic studies using [³H]AMPA and [³H]kainate revealed a differential distribution of sites. [³H]kainate bound to high (K_D = 5 nM) and low (K_D = 27-72 nM) affinity sites within the vertebrate CNS. Distribution of the high affinity kainate binding site corresponded well to the brain regions vulnerable to the neurotoxic action of kainate such as cerebral cortex, the hippocampal CA3 area and the lateral septum, but did not correspond to high affinity [³H]AMPA (Young and Fagg, 1990). High levels of [³H]AMPA binding were seen in the hippocampus, cerebral cortex and the molecular layer of the cerebellum (Young and Fagg, 1990).
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Agonists

L-Glutamate

AMP A

Quisqualate

Kainate

Domoate

Antagonists

DNQX

CNQX

NBQX

Figure 1.7 Structures of agonists and antagonists of the non-NMDA receptors
1.3.5 Molecular Biology of the non-NMDA Receptors

1.3.5.1 Cloning of the non-NMDA receptors

The cloning of the first glutamate receptor subunit in 1989 was a landmark event in the study of these receptors. Due to the difficulties in purifying the glutamate receptors, partial protein sequences could not be obtained. Therefore, a new technique, expression cloning was used (see 1.3.3.1). Utilizing this technique to search a rat brain cDNA library the first glutamate receptor clone, GluR1 was isolated (Hollmann et al., 1989). Using cDNA probes derived from this sequence other related clones were isolated by homology screening and PCR. Molecular cloning has now identified 9 genes encoding subunits of non-NMDA glutamate receptors, GluR1-GluR7 and KA-1 and KA-2 (reviewed in Hollmann and Heinemann, 1994). The AMPA receptor subunits are GluR1-GluR4. The kainate receptor subunits are KA-1 and KA-2, which generate the high affinity $[^3]$H]kainate binding sites and GluR5-GluR7 which are responsible for the low affinity $[^3]$H]kainate binding sites (Bettler and Mulle, 1995).

**GluR1-GluR4 clones**

The GluR1 cDNA has an open reading frame of 2.7 kb and encodes a mature polypeptide sequence of 889 amino acids with a calculated $M_r$ of 99769. This was the largest ligand-gated ion channel subunit then reported, however, the NR2 subunits are now known to be considerably larger (see 1.3.3.1). Hydropathy analysis of the sequence predicted four hydrophobic regions of sufficient length to form transmembrane $\alpha$-helices. Protein sequence comparison with members of the ligand-gated ion channel superfamily, such as nAChR and GABA$_A$R subunits revealed an overall amino acid homology of only $\sim$20%, despite apparent structural homology. The further three clones encoding putative AMPA receptor subunits, GluR2-GluR4, were similar to GluR1. The GluR2-GluR4 cDNAs encoded protein sequences of 862, 866 and 881 amino acids, with predicted
molecular mass of 96400, 98000 and 101034 Da respectively. The overall amino acid sequence identity of GluR1-GluR4 was 70%, see Figure 1.8. These subunits are highly conserved between species, the rat, mouse and human GluR1 subunits have 96-97% amino acid identity. Each of the GluR1-GluR4 subunits has two splice forms, due to alternative splicing of an exon immediately prior the M4 region (Figure 1.8). The alternative exons have been termed 'flip' and 'flop' (reviewed in Hollmann and Heinemann, 1994). A further splice variant for the GluR4 subunit, GluR4c, which is due to an alternative C-terminus of 38 amino acids has been described (Gallo et al., 1992). The alternative C-terminus can exist with either the 'flip' or 'flop' form. A further mechanism for creating diversity is RNA editing. The genes for GluR1-GluR4 all encode a glutamine (Q) residue in the putative M2 region (Figure 1.8). However, for all the GluR2 cDNA clones from adult animals analysed, the glutamine residue at position 582 was replaced by an arginine (R) (Sommer et al., 1991). The selective RNA editing is due to a specific intron sequence in the GluR2 gene (Egebjerg et al., 1994). A proposed mechanism is; a dsRNA structure forms creating a substrate for a dsRNA-specific adenosine deaminase. This enzyme could convert the adenosine to inosine, which could serve as a template for the incorporation of cytosine by reverse transcriptase. Thus the codon would be changed from CAA to CAG i.e. Q to R (Rueter et al., 1995).

GluR5-GluR7 clones

Using low-stringency hybridization screening with probes derived from the GluR1-GluR4 clones and PCR amplification with degenerate primers the first of the kainate receptor subunit cDNAs, GluR5 was cloned (Bettler et al., 1990). This was followed by the cloning of the GluR6 and GluR7 subunit cDNAs (Egebjerg et al., 1991; Bettler et al., 1992). The cDNAs encode for proteins of approximately 900 amino acids and M, ~ 100000. The GluR5, GluR6 and GluR7 subunits share 70-80% amino acid identity with each other, but only ~ 40% with the GluR1-GluR4 subunits. As with the GluR1-GluR4 subunits, 4 membrane
A comparison of the polypeptide sequences encoded by the rat AMPA receptor cDNAs, GluR1-GluR4

Identical amino acids are represented by (*), conservative substitutions are indicated by (.). The putative membrane regions, M1-M4, are shown by ( ). The flip/flop segment is shown by ( —— ). The signal peptide and Q/R site are indicated. The sequence alignment was performed by PC/gene software package, using the method of Higgins and Sharpe (1988).
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leading peptide

GluR1 MFPYFA----FCTGFGLGAV AANFPN1Q1GGLFQNYQGHEAAARFRALSQTLTE 33
GluR2 M-QKHMISWLL4PSWGQSLVF V-SSNHIQ1GGLFQNTGQSAFVSQVQST 32
GluR3 MQGVSRLAVFLL4GLLHSHG G-FNPNTIS1GGLMNTNTVQHSRAFQVQSYNT 32
GluR4 M-R1CROQ1VLLFGFWGGLAMG A-FPSSV1Q1GGLFQNTDQTYFAFLAFLHNT 32

GluR1 FP-------KLLPQIDIVNISDTFEMTYRSQFSDKYAIAGYERTVMMNLT 81
GluR2 FCGLHVCFIPSYFVPSQDTSNQFLQRQPIEAAIHGSEILRQLETDDQR 136
GluR3 FCGALHTSFPSFPTDADVQFVIQMRPALKGAILSLSYKWEKKFVYILDEGER 142
GluR4 FCGALHTSFPSFPTDADVQFVIQMRPALKGAILSIGKWEKCFVYILDEGER 142

GluR1 V-SSNSIQIGGLFPRGADQEYSAFRVGMVQFST 32
GluR2 G-FPNTISIGGLFMRNTVQEHSAFRFAVQLYNT 32
GluR3 G-FPNTISIGGLFMRNTVQEHSAFRFAVQLYNT 32
GluR4 G-FPNTISIGGLFMRNTVQEHSAFRFAVQLYNT 32
M2  M3

**GluR1**  NSLWFSLGAFMQGCDISPRSLSGRIVGGVWWFFTLIIISSYANTLAAFLVERM  625
**GluR2**  NSLWFSLGAFMQGCDISPRSLSGRIVGGVWWFFTLIIISSYANTLAAFLVERM  629
**GluR3**  NSLWFSLGAFMQGCDISPRSLSGRIVGGVWWFFTLIIISSYANTLAAFLVERM  633
**GluR4**  NSLWFSLGAFMQGCDISPRSLSGRIVGGVWWFFTLIIISSYANTLAAFLVERM  630

Q/R site

**GluR1**  VSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFEKMWTYMKSAEPSVFVL  680
**GluR2**  VSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVFDKMWTYMRSAEPSVFVR  684
**GluR3**  VSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVYEKMWSYMKSAEPSVFTR  688
**GluR4**  VSPIESAEDLAKQTEIAYGTLEAGSTKEFFRRSKIAVYEKMWTYMRSAEPSVFTR  692

"flip or flop" segment

**GluR1**  SALRNPVNLAVLKLNEQGLLDKLKNKWWYDKGECGTGGGDSKDKTSALSLSNVAG  790
**GluR2**  SSLGNAVNLAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKEKTSALSLSNVAG  794
**GluR3**  SALGNAVNLAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKDKTSALSLSNVAG  798
**GluR4**  SSLGNAVNLAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKDKTSALSLSNVAG  802

M4

**GluR1**  VFYILIGGLGLAMVLEFCYKSRSESKRMKFGFCILIPQSINEAIRTSSTPRNS  845
**GluR2**  VFYILVGGGLAMVLEFCYKSRAEAKRMK---------------------------  826
**GluR3**  VFYILVGGGLAMVLEFCYKSRSAESKRKMA-------------LT-------------  832
**GluR4**  VFYILVGGGLAMVLEFCYKSRSAEAKRMK--------------------------LTFSEATRNK  837

59
regions are proposed. Both the GluR5 and GluR6 clones are RNA edited at a homologous position to the GluR2 cDNA, although not completely, approximately 39% of GluR5 cDNA and 75% of GluR6 cDNA was found to be in the edited (R) form. The GluR5 clone also has several splice variants, due to the presence or absence of an exon in the N-terminal domain and in the presence or absence of exons in the C-terminus. The N-terminal and C-terminal splice variations can occur together and can be RNA edited or not (reviewed in Hollmann and Heinemann, 1994).

KA-1 and KA-2 clones

Two high affinity kainate receptor subunits have been cloned, KA-1 and KA-2, the cDNAs encoded for polypeptide sequences of 936 and 965 amino acids respectively. They share the same overall structure as the GluR1-GluR7 subunits. However, while KA-1 and KA-2 share 70% amino acid identity, with the GluR5-GluR7 subunits with the GluR1-GluR4 subunits they have only ~ 43% and ~ 37% amino acid identity respectively. Splice variants have not been reported for KA-1 or KA-2, nor is there any indication to date of RNA editing. Both subunits contain a Q at the homologous M2 site (reviewed in Hollmann and Heinemann, 1994).

Subunit topology

The original topology of the GluR subunits was proposed to be four transmembrane regions, due to potential similarity to the nAChR, with the N-terminus extracellular and the C-terminus intracellular, see Figure 1.4 Model 1. However, as for the NMDA receptor subunits (see 1.3.3.1) this proposed topology increasingly appears incorrect. Studies suggest that the domain between the M3 and M4 region is extracellular. In the GluR6 subunit, a consensus glycosylation site was found to be glycosylated in vivo (Roche et al., 1994). Hollmann et al. (1994) also demonstrated N-glycosylation of the same domain in the GluR1 subunit when expressed in Xenopus oocytes. The extracellular location of the M3-M4 domain implied an intracellular C-terminal domain. This
was further supported by the immunocytochemical studies on the GluR1 subunit (Martin et al., 1993; Molnar et al., 1993). Wo and Oswald (1994) demonstrated that the removal of the M2 region did not alter the topology of the mutated kainate receptor subunit as indicated by N-glycosylation. Thus the M2 region did not span the membrane. Bennett and Dingledine (1995) used an epitope protection assay to investigate the topology of the GluR3 subunit. This technique demonstrated an intracellular location for the C-terminus, the extracellular location of the flip/flop region and that both ends of the proposed M2 region were intracellular. The resulting topological model for the glutamate subunits from these studies and studies carried out on the NMDA receptor subunits (see 1.3.3.1) is shown in Figure 1.4, model 2.

1.3.5.2 Distribution of non-NMDA receptor subunit mRNAs

*In situ* hybridization studies have revealed a widespread distribution of the AMPA and kainate receptor subunit mRNAs. However, the individual subunit mRNAs show a more defined pattern of expression.

**GluR1-GluR4 subunit mRNAs**

GluR2 mRNA was found to be almost ubiquitously expressed throughout the brain. However, the expression of the GluR1, GluR3 and GluR4 was more varied. For example, cerebellar granule cells contain abundant GluR2 and GluR4 mRNA, but little if any GluR1 or GluR3 mRNA. In Purkinje cells, there was expression of GluR1-GluR4 mRNA. However, in the cerebral cortex GluR2 mRNA was detected in all layers while GluR1, GluR3 and GluR4 expression differed among the layers. In contrast, the brainstem appears to contain only GluR4 mRNA (reviewed in Hollmann and Heinemann, 1994). The flip and flop variants also had distinct expression patterns, particularly in the hippocampus. CA3 pyramidal cells expressed only the flip form, while CA1 neurons expressed both splice variants of all four subunits. The dentate gyrus had a higher expression of the flop variant relative to the flip form (Sommer *et al.*, 1990).
Differences during brain development were also observed. The flip forms were predominantly expressed in the prenatal rat brain, while postnatally the flip and flop variants were expressed (Monyer et al., 1991).

**GluR5-GluR7 subunit mRNAs**

*In situ* hybridization studies revealed distinct regional differences in GluR5, GluR6 and GluR7 subunit mRNA expression. Moreover, the distribution of the GluR5-GluR7 mRNA is distinct from, more restricted and generally lower than the GluR1-GluR4 mRNA distribution. In cerebellar granule cells there was found to be low levels of expression of GluR5 mRNA, high levels of GluR6 mRNA and an absence of GluR7 mRNA. A similar pattern of expression was seen in the hippocampus. The neocortex had high levels of GluR5 and GluR7 mRNA expression and low levels of GluR6 mRNA (reviewed in Hollmann and Heinemann, 1994).

The expression pattern of GluR6 and GluR7 mRNA correlate with areas known to be especially vulnerable to kainate- and domoate-induced neurotoxicity, such as the cerebral cortex, striatum, hippocampus and cerebellum. Their expression pattern also overlaps with most of the $[^3H]$kainate binding sites (Bettler et al., 1992).

**KA-1 and KA-2 subunit mRNAs**

*In situ* hybridization studies revealed a very distinct distribution of the KA-1 and KA-2 subunit mRNAs. The KA-1 mRNA was generally expressed at low levels and was abundant in only CA3 pyramidal cells and dentate granule cells of the hippocampus. In contrast, KA-2 mRNA expression was found to be generally abundant and widespread, an exception was the thalamus which expressed only low levels of KA-2 mRNA (reviewed in Hollmann and Heinemann, 1994).
1.3.5.3 Immunocytochemical localization of the non-NMDA receptor subunits

Analysis of rat brain sections with subunit-specific antibodies against the GluR1, GluR2/3 and GluR4 subunits showed that the four subunits are abundantly and differentially distributed. The overall pattern of immunostaining for the GluR1-GluR4 subunits correlated well to the *in situ* hybridization studies on the mRNA distribution. The GluR1 antibody showed densely and lightly stained structures, the GluR2/3 antibody gave widespread dense staining and moderate staining was seen with the GluR4 antibody. The Bergmann glia cells showed dense staining with the GluR1 and GluR4 antibodies while cerebellar neurones other than granule cells were most densely stained with the GluR2/3 antibody. Purkinje cells had dense staining with the GluR2/3 antibody and light staining with the GluR1 and GluR4 antibodies. A discrepancy from the *in situ* hybridization studies was the staining of pyramidal and non-pyramidal cells in the CA3 region with the GluR4 antibody. Immunolabelling of the cell body and dendrites was seen. Receptors on the plasma membrane were localized at the postsynaptic densities. There was no evidence for a nonsynaptic or presynaptic localization (Petralia and Wenthold, 1992).

Immunostaining in sections of rat brain with antibodies against GluR6 and KA-2 subunits gave similar patterns with both antibodies. The antibody localization was consistent with the *in situ* hybridization studies. Both antibodies gave significant levels of staining in the olfactory bulb, cerebral cortex, hippocampus and cerebellum. Immunostaining appeared to be localized postsynaptically, similar to that seen for the AMPA receptor subunits. (Wenthold *et al.*, 1994).

1.3.5.4 Biochemical characterization of native non-NMDA receptors

The AMPA receptor complex was solubilized in a non-denatured form from rat brain. Subunit-specific antibodies co-precipitated all four of the GluR1-GluR4 subunits. This suggested that each of GluR1-GluR4 subunits can form a
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heterologous complex with all of the other subunits of this family (Wenthold et al., 1992). Studies to determine the size of the complete AMPA receptor complex gave molecular weight estimates of 610 kDa and 590 kDa (Blackstone et al., 1992; Wenthold et al., 1992). These values are consistent with the proposed pentameric stoichiometry, based on comparison with other ligand-gated ion channels, such as the nAChR. Wenthold et al. (1994) also demonstrated the co-existence of GluR6 and KA-2 subunits in vivo, by co-immunoprecipitation studies using subunit specific antibodies.

1.3.5.5 Properties of recombinant non-NMDA receptors

Following the cloning of the non-NMDA receptors, expression systems were used to determine the pharmacological and functional properties of the non-NMDA subunits. Expression of single subunits and combinations of subunits was carried out to elucidate their effects on receptor function.

GluR1-GluR4 subunits

The GluR1-GluR4 subunits can form functional homomeric AMPA receptor channels following expression in Xenopus oocytes and HEK 293 cells. The order of agonist potencies for electrophysiological studies and for [3H]AMPA radioligand binding studies was quisqualate > domoate ~ AMPA > glutamate > kainate. For homomeric GluR1 receptor channels expressed in Xenopus oocytes, the EC50 values were 0.15 μM, 1.3 μM, 9.2 μM and 39 μM for quisqualate, AMPA, glutamate and kainate respectively (Hollmann et al., 1989; Nakanishi et al., 1990; Sakimura et al., 1990). Kainate and domoate were full agonists while AMPA, glutamate and quisqualate acted as partial agonists. AMPA and glutamate caused rapid desensitization of the recombinant AMPA receptors. GluR2 and GluR4 desensitization time constants are ~36 ms and ~8 ms respectively. Kainate generates larger steady-state currents because it does not desensitize the GluR1-GluR4 receptors (reviewed in Hollmann and Heinemann, 1994). Expression of GluR1, GluR3 or GluR4 in Xenopus oocytes or HEK 293
cells gave receptors which displayed strong inward rectification and were Ca\(^{2+}\)-permeable. However, GluR2 channels produced only small currents with a linear I/V relationship which were Ca\(^{2+}\) impermeable (Hollmann et al., 1991; Burnashev et al., 1995). The difference in channel properties of the GluR2 receptor as compared to the GluR1, GluR3 or GluR4 receptors is due to the RNA edited position in the M2 region (see 1.3.5.1 and Figure 1.8). When the positively charged arginine residue (R586) of GluR2 was changed to the glutamine residue (Q) present in the homologous position in the GluR1, GluR3 and GluR4 subunits the mutant GluR2 channel was Ca\(^{2+}\) permeable and inwardly rectifying. Alternatively if the reverse mutation was carried out on either of the GluR1, GluR3 or GluR4 subunits the resultant mutant channel was Ca\(^{2+}\)-impermeable and had linear a I/V relationship (reviewed in Hollmann and Heinemann, 1994).

The co-expression of two or more GluR1-GluR4 subunits did not alter the order of agonist potency, although the respective potencies were usually lowered. However, heteromeric receptors had larger current amplitudes. Heteromeric receptors composed of GluR1, GluR3 or GluR4 are Ca\(^{2+}\) permeable and inwardly rectifying. The presence of edited GluR2 subunits (see 1.3.5.1) dominated the channel properties, forming receptors which were Ca\(^{2+}\) impermeable and had a linear I/V relationship.

The flip or flop splice variants (see 1.3.5.1) did not affect the pharmacological profile of the heteromeric receptors, nor was Ca\(^{2+}\) permeability, desensitization properties or the I/V relationship altered. However, 'flop' receptors generated current amplitudes 4-5-fold smaller than 'flip' receptors when activated by glutamate, but not when activated by kainate (reviewed in Hollmann and Heinemann, 1994).

**GluR5-GluR7 subunits**

When transiently expressed in HEK 293 cells homomeric GluR5, GluR6 and GluR7 receptors exhibited a kainate binding site, with K\(_D\)s for [\(^3\)H]kainate...
of 70 nM, 95 nM and 77 nM respectively (Bettler et al., 1992; Sommer et al., 1992). Displacement of [³H]kainate binding showed some differences in order of affinity, for GluR5 homomeric receptors the rank order was domoate > quisqualate ~ glutamate > CNQX. For GluR6 homomeric receptors, domoate > quisqualate > glutamate ~ CNQX and for GluR7 homomeric receptors the rank order of affinity was domoate > CNQX > glutamate > quisqualate. However, none of the receptors showed significant AMPA binding. The splice forms of GluR5 (see 1.3.5.1) did not alter the pharmacological profile seen in the radioligand binding studies (reviewed in Hollmann and Heinemann, 1994).

Homomeric expression of unedited GluR5 (see 1.3.5.1) in Xenopus oocytes gave small responses to glutamate, whereas in HEK 293 cells larger responses were seen, with an order of potency of domoate > kainate > glutamate > AMPA. The edited form could not produce a functional homomeric receptor. However, co-expression with the unedited variant altered the I/V relationship from inwardly rectifying to linear. The edited form of GluR6 when expressed in Xenopus oocytes was activated by domoate > kainate > quisqualate > glutamate. There was no response to AMPA. The I/V curves of the edited GluR6 channel are linear, while the unedited form showed inwardly rectifying I/V curves. Both edited and unedited forms of GluR6 are permeable to Ca²⁺ however, the edited form was approximately 3-fold less permeable. Homomeric expression of the GluR7 subunit in Xenopus oocytes or transfected cells did not produce a functional receptor. Co-expression of GluR5-GluR7 with the GluR1-GluR4 subunits did not generate responses which differed from the respective single subunit, indicating that interaction between these receptor subunits was unlikely (reviewed in Hollmann and Heinemann, 1994).

KA-1 and KA-2 subunits

The KA-1 and KA-2 subunits failed to produce functional homomeric channels when expressed in Xenopus oocytes or transfected cell lines. However, [³H]kainate ligand binding studies on transfected cells demonstrated the presence
of high affinity kainate binding sites, with $K_D$ values of 4.7 nM and 15 nM for KA-1 clone and KA-2 clone transfected cells respectively. The order of binding affinity was kainate $>$ quisqualate $>$ domoate $>$ glutamate. There was no detectable $[^3H]$AMPA binding.

Co-expression of the KA-2 subunit with GluR5 or GluR6, both edited and unedited forms, generated functional, agonist-sensitive channels. The rectification properties of heteromeric KA-2/GluR5 or KA-2/GluR6 receptors was determined by the editing at the Q/R site in the M2 region of the GluR5 or GluR6 subunits. The edited subunits generated linear I/V curves, while unedited subunits gave inwardly rectifying I/V curves. Interestingly the KA-2/GluR6 receptor channels responded to AMPA, a property not seen with homomeric GluR6 channels. The co-expression of subunits also altered the $[^3H]$kainate binding properties. The heteromeric KA-2/GluR5 bound $[^3H]$kainate with a $K_D$ of 90 nM. The homomeric subunits bound $[^3H]$kainate with $K_D$ values of 15 nM and 70 nM for KA-2 and GluR5 respectively (reviewed in Hollmann and Heinemann, 1994).

1.3.6 Metabotropic Glutamate Receptors

In the 1980s it became apparent that glutamate could stimulate phospholipase C (PLC) and phosphoinositide metabolism via a receptor which was not part of the identified ligand-gated ion channel glutamate receptor family (e.g. Sladeczek et al., 1985; Nicoletti et al., 1986). The studies suggested that glutamate was activating receptors coupled to G-proteins. This was confirmed by studies in Xenopus oocytes using rat brain mRNA (Sugiyama et al., 1987). Further work also demonstrated mGluRs which were negatively coupled to adenylyl cyclase (Prézeau et al., 1992).

A number of agonists for mGluR were identified, including quisqualate and ibotenate which also acted on the ionotropic glutamate receptors. Specific agonists for mGluRs included 1-aminocyclopentane-1,3-dicarboxylate (ACPD),
2-(carboxycyclopropyl)glycine (CCG) and L-2-amino-4-phosphonobutyrate (L-AP4). Antagonists for mGluRs included α-methyl-4-carboxyphenylglycine (MCPG) and L-2-amino-3-phosphopropionic acid (L-AP3) (reviewed in Pin and Duvoisin, 1995).

The first mGluR was cloned by functional expression in *Xenopus* oocytes (Houamed *et al.*, 1991; Masu *et al.*, 1991). Low stringency hybridization or PCR using degenerate primers isolated a further seven genes for mGluRs and several splice variants. Based on sequence identity the 8 mGluRs were sub-divided into 3 groups; Group I contained mGluR1a-e and mGluR5a-b; Group II comprised mGluR2 and mGluR3; while mGluR4a-b, mGluR6, mGluR7 and mGluR8 comprised Group III. The deduced amino acid sequences of these receptors did not share any sequence homology with previously known G-protein-coupled receptors. The mGluR1a receptor was found to be 1199 residues in length, considerably larger than previously known G-protein-coupled receptors. The proposed topology has large N- and C-terminal domains and 7 transmembrane regions (reviewed in Pin and Duvoisin, 1995). The transduction mechanisms of the cloned mGluRs were identified following transfection into various expression systems. These concurred with the amino acid identity subdivisions. Group I receptors stimulated PLC, resulting in increased phosphoinositide metabolism and Ca\(^{2+}\) release from internal stores. Group II and Group III mGluRs were negatively coupled to adenyl cyclase. The most potent agonists for the cloned mGluRs were quisqualate, (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I) and L-AP4 for Group I, Group II and Group III respectively (reviewed in Pin and Duvoisin, 1995).

*In situ* hybridization studies have demonstrated the widespread location of mGluR mRNA throughout the brain. However, individual genes have a more localized expression (e.g. Shigemoto *et al.*, 1992; Ohishi *et al.*, 1993; Nakajima *et al.*, 1993; Tanabe *et al.*, 1993; Okamoto *et al.*, 1994). Various subtypes of mGluRs have been shown to modulate voltage-sensitive Ca\(^{2+}\) channels, K\(^{+}\)
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channels and ionotropic receptors such as GABA, NMDA and AMPA receptors. It has also been proposed that mGluRs play a role in synaptic plasticity, in both LTP and LTD (reviewed in Pin and Duvoisin, 1995).

1.4 LTP AND MEMORY

LTP is the persistent enhancement of synaptic strength that is observed after high-frequency stimulation of a population of neurons. This effect is believed to underlie at least some forms of memory and was first described in detail in 1973 (Bliss and Lomo, 1973; Bliss and Gardener-Medwin, 1973; reviewed in Bliss and Collingridge, 1993). The primary model of LTP has been the CA1 region of the hippocampus, although since the original observations, LTP has been found in other areas and excitatory pathways of the brain (e.g. Bashir and Collingridge, 1992; Clugnet and LeDoux, 1990; Squire, 1986).

In 1949, Hebb proposed a coincidence-detection rule; the synapse uniting two neurons would be strengthened only if the pre- and post-synaptic cells were active at the same time. The NMDA receptor was identified as the key component of this scheme - the 'molecular-coincidence detector' - by antagonist studies. AP5 was shown to prevent induction of LTP (Collingridge et al., 1983). Moreover, in 1984, Mg^2+-antagonism of NMDA receptors was shown to operate in a voltage-dependent manner (Nowak et al., 1984). Therefore NMDA channel activation required both presynaptic activity to release L-glutamate and postsynaptic depolarization to reduce the Mg^2+ block, thus demonstrating the requisite properties to be the molecular-coincidence detector (reviewed in Bliss and Collingridge, 1993). Electrophysiological studies have demonstrated the co-localization of NMDA receptors and non-NMDA receptors at the same synapse (Bekkers and Stevens, 1989) thus allowing post-synaptic depolarization following glutamate release. It is assumed that the Ca^{2+} influx through NMDA channels provides the signal for induction of LTP. The events maintaining induction of
LTP are less clear, protein kinases have been implicated as have the mGluRs (Bashir et al., 1993). There is also debate over the locus of expression of LTP. Evidence for post-synaptic changes, such as insertion of new or additional receptors in the membrane and pre-synaptic phenomenon such as increased transmitter release has been demonstrated. Other models have suggested both pre-synaptic and post-synaptic modifications are involved (Edwards, 1995).

Studies of LTP and neuronal development have recently been made with mice which lack one of the NMDA receptor genes (Li et al., 1994; Sakimura et al., 1995; Ebralidze et al., 1995). Gene targeting to the NR1 gene prevented the development of functional NMDA receptors. This affected the development of neural patterns and was a lethal mutation with the mice dying 10-20 h after birth (Li et al., 1994). Mice lacking the NR2A subunit developed normally, although their behaviour was affected. They generally performed less well in spatial learning tasks and hippocampal LTP was reduced but not abolished (Sakimura et al., 1995). The NR2C gene 'knockout mice' also developed normally, nor did they show any gross behavioural or motor differences from wild-type mice. Patch-clamping studies on cerebellar granule cells from mature NR2C knockout mice showed only high conductance states (42 pS), which were similar to those seen from immature (under P12) wild-type mice when no NR2C subunit is expressed. The mutant mice lacked the low conductance states (~30 pS) of mature, wild-type mice cerebellar granule cells. However, the NR2C subunit-lacking mutant mice did not show any significant differences in the performance of spatial learning tasks, such as the water maze, as compared to wild-type mice (Ebralidze et al., 1995).
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1.5 GLUTAMATE PATHOPHYSIOLOGY

Work by Olney and colleagues suggested that glutamate could act as an excitotoxin, as well as a neurotransmitter (reviewed in Olney, 1990). It was demonstrated that systemic administration of glutamate and other excitatory amino acids to brain regions caused a characteristic cytopathology. The postsynaptic structures (dendrites and somas) were destroyed but axons, pre-synaptic terminals and non-neuronal cells survived. Structure-activity studies showed a correlation between the neurotoxic potency and excitatory potency of different excitatory amino acid analogues.

Development of specific agonists for the ionotropic glutamate receptor, NMDA, kainate and AMPA allowed the study of their role in neurotoxicity (Watkins et al., 1990). Neurons showed variation in their vulnerability to different agonists. Moreover, in some cases this variation reflected the pattern of receptor expression. In vivo studies of glutamate toxicity were difficult due to the active glutamate uptake systems, necessitating high concentrations of exogenous glutamate. However, tissue culture studies using antagonists gave variable results. Some studies indicated that NMDA receptors were the main mediators of neurotoxicity (Schramm et al., 1990; Lombardi et al., 1993), while others implicated non-NMDA receptors (Mattson et al., 1989; Frandsen et al., 1990), yet other culture studies demonstrated the involvement of both NMDA and non-NMDA receptors (Schousboe et al., 1992; Frandsen and Schousboe, 1993). Tissue culture studies identified two processes of neurotoxicity, acute and delayed, following prolonged exposure to micromolar concentrations of glutamate (Meldrum and Garthwaite, 1990). However, it was probable that the toxic mechanism of excessive Ca\(^{2+}\) influx was common to both. Release of Ca\(^{2+}\) from internal stores also contributed to NMDA receptor-mediated neurotoxicity, but not AMPA- or kainate- induced toxicity (Frandsen and Schousboe, 1993). The direct correlation between cell death and intracellular Ca\(^{2+}\) concentration
does not always hold. While intracellular Ca\(^{2+}\) concentrations rise to several times basal levels in neurons exposed to toxic levels of glutamate or NMDA, other procedures which also raised the intracellular Ca\(^{2+}\) levels showed little toxicity (Dubinsky and Rothman, 1991; Tymianski \textit{et al.}, 1993). The location of the Ca\(^{2+}\) influx and concentration increase may be important, possibly in activating Ca\(^{2+}\)-sensitive enzymes (Rothman and Olney, 1995).

NMDA receptor-mediated toxicity was the most well characterized. Several types of NMDA receptor antagonist were shown to prevent NMDA toxicity. These included competitive antagonists such as AP5 and CPP, glycine site antagonists, e.g. HA-966 and the channel blocking compounds, MK801 and ketamine. Acute NMDA toxicity was prevented only when antagonist was co-administered with the agonist (Chen \textit{et al.}, 1992). Delayed NMDA toxicity induced a gradual neuronal degeneration over several hours, which was inhibited by delayed administration of antagonists. Delayed NMDA mediated toxicity was also prevented by pre-incubation with sublethal concentrations of NMDA; this effect required \textit{de novo} RNA and protein synthesis (Chaung \textit{et al.}, 1992; Marini and Paul, 1992). Studies using NMDA receptor antagonists have implied a role for NMDA receptors in the neuronal cell death following status epilepticus, hypoglycaemia and focal ischaemia (Meldrum and Garthwaite, 1990). Clinical trials of a NMDA receptor antagonist for treatment following stroke are proceeding (Grotta, 1994).

Non-NMDA induced toxicity can be mediated by AMPA or kainate receptors (Meldrum and Garthwaite, 1990). Domoate poisoning, due to eating contaminated sea-food causes symptoms including convulsions and amnesia and is a result of excessive activation of non-NMDA receptors (e.g. Tasher \textit{et al.}, 1991). Domoate is an analogue of kainate with approximately a three-fold greater potency, like kainate it is synthesised by a seaweed (Meldrum and Garthwaite, 1990). Non-NMDA receptor antagonists are effective in preventing neuronal damage in models of global ischemia (Sheardown \textit{et al.}, 1990; Kaku \textit{et al.},
1991). They may also protect against certain forms of epilepsy (Turski et al., 1992).

The involvement of glutamate neurotoxicity has also been proposed for some chronic neurodegenerative diseases, for example, Huntington's disease, amyotrophic lateral sclerosis, Parkinsonism and Alzheimer type senile dementia (Dure et al., 1991; Krogsgaard-Larsen et al., 1993).

1.6 AIMS OF THIS STUDY

L-glutamate is the major excitatory neurotransmitter in the mammalian CNS. Five genes have been cloned which encode the subunits of the NMDA subclass of the glutamate receptor, NR1 and NR2A-NR2D. The main aim of this study was to establish a recombinant model system for the cerebellar type NMDA receptor population by transient expression of the respective subunits in the HEK 293 cell line. Firstly, the characterization of native mouse cerebellar NMDA receptors by radioligand binding was described. Secondly, the subcloning of the cDNA encoding the NR2C subunit into a mammalian expression vector and the characterization of recombinant heteromeric NMDA receptors by immunoblotting and radioligand binding was reported. As the co-expression of NR1 and NR2C subunits resulted in a receptor with a low affinity for $[^3H]MK801$, site-directed in vitro mutagenesis was used to try and identify residues in the channel-forming M2 domain important for high affinity MK801 binding.
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Transient expression of cloned heteromeric NMDA receptors in mammalian cells
CHAPTER 2

2.1 INTRODUCTION

2.1.1 Principles of Radioligand Binding to Receptor Populations

Receptors are proteins which mediate the cellular effects of intercellular messengers such as neurotransmitters, hormones and growth factors. They are generally located on the cell surface, exceptions are the steroid and thyroid hormone receptors which are found within the cell nucleus. Receptors function via the capacity to recognise and bind specific signals, known as ligands. Implicit in this, is the assumption that the receptor-ligand complex is responsible for the production of a biological response. This complex is created through a reversible, equilibrium reaction;

\[ R + L \neq RL \]

Where R is the receptor, L is the free ligand and RL represents the receptor-ligand complex. Studies investigating this equilibrium use very low concentrations of ligand, necessitating the radioactive labelling of the ligand. The aim of radioligand binding studies is to investigate the specific interaction between the radioactively labelled ligand and its receptor. From such studies the values for the dissociation constant \((K_D)\) and maximal binding \((B_{MAX})\) can be determined. These quantities are directly analogous to the \(K_M\) and \(V_{MAX}\) values of enzyme kinetics. To measure the extent of binding, it is necessary to separate the bound ligand in the receptor-ligand complex from the free ligand without disturbing the equilibrium between the two states.

The basic technical requirements for such studies on any receptor population are; a tissue preparation containing the receptor of interest, a specific, high-affinity radiolabelled ligand and a method for separating the free and bound ligand. However, a number of criteria must be fulfilled to ensure that any observed binding is actually demonstrating interaction between the radioligand and the receptor of interest. The binding should be target-cell specific. For many systems only some cell types respond to a given signal. Therefore the receptors
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should exist on or in these tissues, but not elsewhere at the same density. However, this is not applicable to all systems, an example is the insulin receptor which appears to be present on almost all cells. The receptor population should have a finite number of binding sites, thus the radioligand binding should be saturable. Saturation is demonstrated by a constant $B_{\text{MAX}}$ value above a certain radioligand concentration. The affinity of the radioligand for the receptor preparation should be appropriate to the concentration range in which the ligand gives a biological response \textit{in vivo}. The binding should demonstrate specificity, biologically active, (+) stereoisomer forms of ligands should be more potent than their counterparts and analogues of the ligand which are not biologically active should not displace the bound radioligand.

Further consideration of the radioligand is also necessary. The basic requirements of the radioligand are that it is biologically active, chemically pure and metabolically stable. The endogenous ligand is not commonly used, since there is the possibility of contamination of the receptor preparation from \textit{in vivo} sources and there are often active mechanisms for the removal or breakdown of the endogenous messenger. Moreover, many endogenous ligands bind to and activate multiple receptor subtypes. This gives a complex binding pattern that is difficult to interpret. Preferably, the radioligand should have a high affinity for the receptor sites, thus allowing the use of low concentrations of radioligand and have a high specific radioactivity as the absolute number of binding sites for many receptor populations is low. Both tritiated and iodinated ligands have been used for binding studies. Tritiated ligands have a long radioactive half-life and no alteration of the chemical structure of the ligand. Iodinated ligands have a much higher specific activity. Incorporation of a single atom of $^{131}\text{I}$ or $^{125}\text{I}$ in a molecule would give a specific activity of 16250 Ci/mmol or 2175 Ci/mmol respectively. Whereas incorporation of a single $^3\text{H}$ per molecule would give a specific activity of 29 Ci/mmol. However, iodination can potentially alter the biological activity of the ligand. The iodine isotopes also have relatively short
half-lives, 8 days for $^{131}$I and 60 days for $^{125}$I.

To quantify the radioligand binding, the bound and free radioligand must be separated without significantly altering the equilibrium between the two states. A number of techniques have been used, which vary according to their ease of operation, speed of operation (rate of separation of bound from free ligand) and the reproducibility of the results. The technique chosen will depend upon the rate of dissociation of the ligand from receptor. Filtration is the most commonly used technique. It is rapid with a separation time of approximately 10 s, simple to perform, gives good separation of bound and free ligand and good reproducibility of results. The incubation volume is rapidly filtered through a matrix, such as a glass-fibre filter which traps the receptor-ligand complex. The filters are then washed with cold buffer to remove any free ligand which may be trapped. Another technique for separation is centrifugation. This does not alter the equilibrium conditions within the radioligand binding assay. However, it takes longer and the specific: non-specific binding ratio is poor, possibly due to trapping of free ligand within the pellet of protein.

The interpretation of binding data is complicated by the presence of non-specific binding. This is radioligand that is absorbed to glass, paper, membranes and other substances other than the specific receptor site within the assay. Non-specific binding is usually non-saturable and it has low affinity. In practice, it is considered to be the radioactivity that is not displaced by relatively high concentrations of a competitive, non-labelled ligand. It is assumed that the competing, non-labelled ligand occupies all the high-affinity receptor sites, but does not interfere with the binding of the radiolabelled ligand to the non-specific sites. Therefore the specific binding, which is radioligand bound to the receptor site, is the total amount of radioactivity on a filter minus the non-specific binding. If the unlabelled, inhibitor ligand is non-identical to the radiolabelled ligand, it is necessary to verify that the inhibitor ligand is behaving competitively, since suppression of radioligand binding does not imply a
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competitive mechanism.

The lowest concentration of the competitor ligand, consistent with 99% displacement of the specific radiolabelled ligand binding should be used. A large excess of the unlabelled ligand may cause displacement of non-specific sites binding. This would generate an incorrect model containing multiple specific sites. If the competitor and radiolabelled ligand are the same, or they have similar $K_D$ values, a 10- and 100-fold excess is sufficient to displace 91% and 99% respectively, of the radiolabelled ligand from the high affinity receptor site. If the dissociation constants for the two ligands are very different, a large molar excess of one may be necessary.

The commonest method for the determination of non-specific binding is direct subtraction. The binding from incubation tubes containing the radiolabelled ligand and the unlabelled competitor is subtracted from the binding within a parallel set of incubation tubes containing only the labelled ligand. This method assumes that the amount of non-specifically bound radiolabelled ligand is the same in both tubes. However, non-specific binding is proportional to the free ligand concentration, which is not the same in both sets of tubes, as the total ligand concentration in each set of incubation tubes is not equal. If the specific binding is much greater than the non-specific binding the problem is minimized. Purification of the receptor and extensive washing after the equilibration of the ligand and receptor site can improve the accuracy of the method. Direct subtraction is the only method if single point analysis is used, that is, the use of a single concentration of radiolabelled ligand.

The problem of allowing for the difference in free ligand in the total binding and non-specific binding incubation tubes can be overcome by using a linear regression estimate of the non-specific binding at the appropriate free ligand concentration. The values for the non-specific binding and free ligand concentration can be used to plot a line of linear regression. The concentration of free ligand in the total binding incubation tubes can be determined and the
appropriate non-specific binding value can be found via the line determined by linear regression. This method assumes that the non-specific binding is linear, for many systems at appropriate competitor ligand concentrations this is so (Peck and Kelner, 1982; Hulme, 1990)

Saturation experiments, using increasing concentrations of the radioligand, or displacement experiments, where a fixed concentration of radioligand is displaced by increasing concentrations of an unlabelled ligand, are used to characterise receptors and their subtypes. The binding data is analysed to yield the affinity constants ($K_D$), inhibition constants ($IC_{50}$ or $K_i$) and maximal binding ($B_{MAX}$) values. Saturation curves are often converted to a Scatchard plot.

$$\frac{RL^*}{L^*} \text{ vs } RL^*$$

Where $RL^*$ is the radioligand bound to the receptor and $L^*$ is the free radioligand concentration, the gradient $=-1/K_D$ and the x-axis interception gives the $B_{MAX}$ value. A linear Scatchard plot implies a non-interacting, homogenous receptor population. Deviations from linearity could result from either heterogenous populations of receptors with different affinities for the radioligand or positive or negative co-operativity between binding sites within the receptor molecule. Alternatively, the apparent deviation may be due to binding artefacts in the assay, for example incomplete equilibration with the radioligand, over or underestimation of the non-specific binding.

Displacement assays are used to investigate the binding of unlabelled compounds on a receptor by their ability to displace a radiolabelled ligand. The concentration of unlabelled ligand required to displace 50% of radioligand binding is the inhibition constant. If the interaction is non-competitive the value is known as the $IC_{50}$. However, if the interaction is competitive with the radioligand, thus dependent upon the concentration of the radioligand in the assay, a $K_i$ value is generated.
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The $K_I$ is related to the concentration and affinity of the radioligand by the Cheng-Prussoff equation.

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

An accurate determination of the radioligand affinities for each receptor subtype in heterogeneous populations of receptors is essential for the determination of inhibition constants by displacement assays and the correct quantification of receptor subtype ratios. However, the ability of such experiments to detect subtypes of receptors is restricted. For a saturation curve to be able to detect two receptor subtypes the $K_D$ values must be sufficiently different, the maximal amounts of the radioligand bound must be within the same order of magnitude and the range of the radioligand concentrations used sufficiently large to encompass both $K_D$ values. Generally the tritiated radioligands are used in the range 0.1-100 nM and iodinated radioligands in the range of 1-1000 pM. To detect two different affinity sites by homologous displacement assays (the radioligand and displacing ligand are the same) the amounts of the radiolabelled ligand bound to the two sites must be similar and the $IC_{50}$ values must be sufficiently different. This is possible if the radioligand concentration used is low and the low affinity sites are in large excess. However, under these conditions the two sites would not be detected by saturation binding. If a high concentration of radioligand was used the displacement curve would appear to show single site behaviour. In many cases a homologous displacement curve would not detect the presence of two binding sites of different affinity. It is also possible that a saturation curve would be unable to detect the complexity. This problem may be surmounted by parallel analysis of binding kinetics and equilibrium binding, however, this is complex. Simpler methods involve parallel binding studies. A saturation assay using the lowest possible radioligand concentration range and in parallel a homologous displacement assay at the lowest radioligand concentration possible. If the midpoints of the two curves are different (the $K_D$ and $IC_{50}$ values respectively), this would suggest that the simple
one site model is incorrect. Alternatively, a series of heterologous displacement curves at different radioligand concentrations could be performed. Any correlation between the radioligand concentration and either the calculated $K_D$ values or the proportions of the sites would indicate complexity (Hulme, 1990; Swillens et al., 1995).

2.1.2 Expression of Foreign DNA in Mammalian Cells

The controlled expression of proteins in *in vitro* systems has aided the determination of protein structures and proved valuable in functional studies and biochemical characterization. There are two major types of expression system available, prokaryotic and eukaryotic. The choice of which system is used is dependent upon the protein to be expressed and the type studies to be carried out upon the expressed protein. Prokaryotic systems do not carry out post-translational modification of the expressed protein. If such modifications are not important for the studies, a prokaryotic system such as *Escherichia coli* (*E. coli*) can be used. However, eukaryotic proteins *in vivo* generally have some form of post-translational modification. Therefore, a eukaryotic system is required to generate a more accurately modified protein. Such systems include yeast cells, insect cells and mammalian cell cultures. The introduction of the foreign DNA into these cells can be direct as in transfection or it can be mediated by viruses, for example, the Simian virus (SV) 40 virus with mammalian cell lines and the baculovirus with insect cell lines have been utilized. Methods for direct transfection of DNA into cells include calcium phosphate co-precipitation (Gorman et al., 1990), diethylaminoethyl-dextran (DEAE-dextran) (Sussman and Milman, 1984), electroporation (Neumann et al., 1982; Chu et al., 1987) and lipofection (Felgner et al., 1987). Cells can be stably transfected with the foreign DNA incorporated into the chromatin of the host cell or by permanent maintenance of the plasmid. A selection system such as neomycin is used to allow only cells which maintain expression of the plasmid DNA to survive. The
alternative procedure is transient transfection. There is no selection for the transfected cells so the foreign DNA is not maintained in the cells for extended periods. Generally the cells are harvested and assayed for the expressed protein 24-72 h following the transfection procedure. Stable expression of the inserted gene is often considered to be preferable since successful stable transfection produces a clonal cell line expressing the gene of interest, giving an unlimited supply of the protein. However, creating stable cell lines can be very labour intensive. Therefore, many studies have been carried out using transient expression systems. These allow studies to be initiated more rapidly, although the level of protein expression is generally lower as only a proportion of the cells will be transfected.

For efficient transient expression of the protein a number of factors need to be considered. These are the number of cells which take up the DNA; the number of copies of foreign cDNA per cell; the processing of the respective mRNAs; the stability of the expressed protein and the transcriptional and translational efficiency. For the study of membrane-bound receptors, the in vivo conditions of the receptor are most closely mimicked by mammalian cell lines. Cell lines with rapid doubling times (<20 h) are preferable since there is a high correlation between the growth rate of cells in culture and the ability to take up DNA (Gorman, 1985). Some possible cell lines include Chinese hamster ovary (CHO) cells, COS cells, HeLa cells and human embryonic kidney (HEK) 293 cells. A primary requirement in the selection of the cell line to be used for transfection studies, is that it does not express the protein of interest. The COS cell line has been extensively used in studies of the nAChR (Verrall and Hall, 1992). This cell line was derived from African Green Monkey Kidney CV-1 cells transformed with an origin defective SV40 virus (Gluzman, 1981). HEK 293 cells have been adenovirus transformed and have a strongly active human cytomegalovirus (CMV) promoter allowing high levels of expression (Gorman et al., 1990). This cell line has been widely used for studies on GABA_A receptors.
(Pritchett et al., 1988), non-NMDA receptors (Sommer et al., 1990) and NMDA receptors (Cik et al., 1993).

Mammalian cell transient expression systems require specially constructed vectors, which must allow growth and replication in eukaryotic and prokaryotic cells. They contain prokaryotic elements such as ColE1 - an origin of replication for any *E.coli* strain, an antibiotic resistance gene for selection and T7 and SP6 RNA promoters for sense and antisense RNA production. Eukaryotic expression requires multiple elements such as an SV40 or Epstein Barr virus origin of replication, promoter and enhancer sequences and mRNA processing signals. Promoters can be inducible or constitutive. They activate gene transcription in an orientation and distance-specific manner. Inducible promoters are regulated and require a chemical signal for activation and are often used in the production of stably transfected cell lines, especially if the expressed protein is toxic to the cells. Constitutive promoters are permanently active. Promoter sequences generally consist of an AT-rich motif and are found upstream of the initiation site of transcription. The 'TATA' box is essential and located 20-25 bp upstream. The 'CAAT' box can also be present and is found 80 bp upstream. Enhancer sequences stimulate gene transcription from virtually any promoter near to them in an orientation and distance non-specific manner. However, many enhancer sequences are tissue specific (Maniatis et al., 1987). Transcription termination, site-specific post-translational cleavage and polyadenylation all require mRNA processing signals. In mammalian expression vectors they are often derived from the SV40 virus. A number of such vectors have been developed, often they contain more than one viral origin of replication to allow wider application of use. The pCIS vector, as shown in Figure 2.3.5, has been used in HEK 293 cells for studies on a variety of proteins; secreted proteins such as relaxin and human growth hormone (Gorman et al., 1990); membrane-bound proteins such as GABA<sub>A</sub> receptors (Pritchett et al., 1988) and glycine receptors (Pribilla et al., 1992). This vector is driven by a CMV promoter-enhancer. It was constructed
by Gorman et al. (1990); SV40 elements are also present, which are a SV40 ori and SV40 mRNA processing signals. The pCIS is readily grown in *E. coli* due to the ColEl element and it is selected for by the ampicillin resistance gene. The M13 ori allows recovery of (+) ssDNA when grown in the presence of M13 helper phage (Vieira and Messing, 1987).

Of the direct DNA transfection methods listed previously, the most widely used is the calcium-phosphate co-precipitation method. The DNA forms an insoluble complex (CaP-DNA) when mixed with calcium phosphate. This precipitate is believed to induce phagocytosis when presented to the cell, facilitating entry of DNA into the cell cytoplasm. The vector is then transferred to the nucleus. The CaP-DNA complex also protects the DNA from nucleases both intracellular and from within the culture media (Loyter *et al.*, 1982). The efficiency of transfection by this method is dependent on cell type. It can be improved by either by glycerol shock of the cells after transfection (Gorman *et al.*, 1990) or by the addition of chloroquinine (Luthman and Magnusson, 1983).

Expression of receptor genes in mammalian cells allows manipulation of the expressed receptor population. Various combinations of subunits can be co-expressed. The subunits of the receptor can be manipulated, for example by site-directed mutagenesis, prior to the expression as part of the recombinant receptor. The biochemical and pharmacological properties of the cloned receptors can be compared to those of the native receptors. Insights into the structure and function of the receptors may be elucidated due to the more controlled situation of recombinant receptors. This strategy has been utilized in the following studies.
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In this chapter, the characterisation of native adult mouse cerebellar NMDA receptors and recombinant NMDA receptors transiently expressed in a mammalian cell line will be described. The clones used were the rat NR1a (also known as NR1-1a) and the mouse NR2A and NR2C clones, due to the restricted and high-levels of expression for the NR2C mRNA and the presence of NR2A mRNA in adult cerebella (Kutsuwada et al., 1992). The NR1a subunit is over 99% identical between rat and mouse, however, the NR2 subunits display greater variation between the two species. Therefore, the cloned receptor studied here is a mouse NMDA receptor. Radioligand binding studies, immunoblotting and cytotoxicity assays were used to characterise and compare the recombinant receptor populations and wild-type mouse NMDA cerebellar receptors.
2.2 MATERIALS AND METHODS

2.2.1 Materials

Restriction enzymes, T4 DNA ligase, calf intestinal alkaline phosphatase (CIP), DNA molecular weight markers II (λ DNA Hind III digested), lysozyme, ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (x-gal) and caesium chloride were from Boehringer Mannheim GmbH., (Lewes, Sussex, U.K.). QIAEX gel extraction kit and QIAGEN plasmid purification kit were obtained from QIAGEN Ltd., (Dorking, Surrey, U.K.). Magic™ DNA clean-up system and the plasmid pSV-β-galactosidase were from Promega Corporation (Southampton, U.K.). Ultrapure agarose and pre-stained molecular weight standards for protein electrophoresis were from Life Technologies Ltd. (Uxbridge, U.K.). Bacterial culture media components (tryptone, tryptone yeast extract broth, bacto-agar) were from Difco Labs., (East Molesley, U.K.). Bacterial strain *E. coli*. INV1α (DH1 derivative, Genotype: endA1, recA1, hsdR17(λ, m^+^), supE44, thi-1, gyrA, relA, Φ80 lacZΔM15Δ(lacZYA-argF), deoR^+, F^+) was obtained from British Bio-technology Ltd., (Oxford, U.K.). Pre-equilibrated phenol (pH > 7.6) was from Fisons (Leicestershire, U.K.). Tissue culture media (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham, DMEM/F12 1:1 mixture), trypsin-EDTA, Hanks' balanced salt solution, ribonuclease A (RNAase A), ethidium bromide and ketamine hydrochloride were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Sterile foetal calf serum (FCS) was from Advanced Protein Products (West Midlands, U.K.). Sterile disposable filter units Sartolab-V150 were from Sartorius (Goettingen, Germany). DL-2-Amino-5-phosphonopentanoic acid (AP5) was purchased from Tocris Neuramin (Essex, U.K.). [^3H]MK801 was from DuPont (Stevenage, Herts, U.K.). MK801 maleate was obtained from Research Biochemicals Inc., (Natick, MA, U.S.A.). Acrylamide and N′N′-methylenebisacrylamide were from BDH Laboratory Supplies (Leicestershire, U.K.). ECL reagents and Hyperfilm™ for
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Western blot detection were from Amersham International plc., (Buckinghamshire, U.K.). The peptide NR1-(911-920), amino acid sequence LQLCSRHRES, was from Multiple Peptide Systems (San Diego, CA.). The peptides CysNR2C-(1-14), NR2C-(1208-1218) and the multiple antigen peptide (MAP), NR2A-(1432-1442)AA, (YKKMPSIESDVAA)-MAP were from Peptide and Protein Research (University of Exeter, Devon, U.K.) The antibodies were raised and characterized by Dr. P. L. Chazot.

Human embryonic kidney (HEK) 293 cells were a gift from Dr. T.G. Smart (School of Pharmacy, London, U.K.). The pCIS plasmid was a gift from Dr. C. Gorman (Genentech, South San Francisco, California, U.S.A.). The plasmid constructs pBKSII+NRc1 and pBKSII+NRc3 (also known as pBKSII+NR2A and pBKSII+NR2C respectively) were generous gifts from Dr. M. Mishina (The School of Medicine, Niigata University, Niigata 951, Japan). The plasmid construct pN60-NR1a was a generous gift from Dr. Shigetada Nakanishi (The Faculty of Medicine, Kyoto University, Kyoto, Japan). Tenocyclidine (TCP) was a gift from Dr. J. F. Collins (City of London Polytechnic, London, U.K.).

All other reagents were from commercial sources.

2.2.2 Storage of E. coli

For long term storage, overnight culture (0.5 ml) was mixed with a 1:1 (v/v) solution of Luria-Bertani (LB) media (tryptone 10 g/l, yeast extract broth 5 g/l and NaCl 10 g/l) and sterile glycerol (0.5 ml) in a sterile cryogenic tube. This was supplemented with ampicillin (50 μg/ml) as necessary and stored at -20°C.
2.2.3 Preparation of Competent Cells

*E. coli* in Media A, which was LB media supplemented with 10 mM MgSO$_4$ and 0.2% (v/v) glucose, (15 ml) were grown overnight at 37°C with vigorous shaking. Overnight stock (0.5 ml) was further grown in fresh media A (50 ml) at 37°C with vigorous shaking in a Luckham R300 orbital shaker at 200 rpm until midlog phase had been reached. This was for approximately 3-4 h when the optical density at $\lambda = 600$ nm was 0.6-0.8. The suspension was incubated on ice for 10 min, then centrifuged at 300 g for 10 min at 4°C. The pellet was gently resuspended in ice-cold Media A (0.5 ml) with a heat-sealed pasteur pipette. Media B, which was LB media supplemented with 36% (w/v) glycine, 12% (w/v) PEG 8000 and 12 mM MgSO$_4$, (2.5 ml) was added and gently mixed with a heat-sealed pasteur pipette. The competent cells were stored at -80°C in 100 μl aliquots. (Nishimura *et al.*, 1990).

2.2.4 Transformation of Competent Cells

A frozen aliquot of competent *E. coli* was thawed on ice for 5 min. The appropriate DNA solution (2-5 μl, 10-50 ng DNA) was added and mixed gently. The mixture was incubated on ice for 30 min, heat-shocked by placing in a waterbath at 42°C for 60 sec followed by incubation on ice for 2 min. LB media (900 μl) was mixed with the transformed *E. coli*. This was incubated at 37°C for 60 min. The appropriate volume (50-300 μl) was plated onto culture dishes prepared with 1.5% (w/v) agar in LB media containing 100 μg/ml ampicillin. These were incubated at 37°C in an inverted position for 15-18 h. (Sambrook *et al.*, 1989).
2.2.5 DNA Plasmid Preparation

2.2.5.1 Small scale preparation

LB media (10 ml) containing ampicillin (100 μg/ml) was inoculated with *E. coli* containing the plasmid of interest and amplified overnight at 37°C with shaking at 200 rpm. Overnight culture (1.5 ml) was aliquoted into microfuge tubes. The suspension was centrifuged at 12000 g for 3 min at room temperature, the supernatant was removed by aspiration and discarded. The pellet was recentrifuged for 30 sec and reaspirated. The pellet was resuspended in ice cold TGE buffer which was, 50 mM Tris-HCl, pH 8.0, 50 mM glucose and 10 mM EDTA (100 μl), alkaline sodium dodecylsulphate (SDS) which was, 200 mM NaOH and 1% (w/v) SDS, (200 μl) was added, followed by ice-cold potassium acetate, 3 M, pH 4.8 (150 μl). The solution was incubated on ice for 5 min, then centrifuged at 12000 g for 5 min at room temperature. The DNA-containing supernatant was transferred to a fresh microfuge tube. The supernatant was extracted with an equal volume of phenol/chloroform mixture (phenol:chloroform:amyl alcohol 25:24:1 (v/v) ratio). The emulsion was centrifuged at 12000 g for 90 sec at room temperature. The upper (aqueous) layer was transferred to a new microfuge tube. The double-stranded (ds) DNA was precipitated by the addition of 96% (v/v) ethanol (2 vol), incubation on ice for 15 min then centrifugation at 12000 g for 15 min at 4°C. The supernatant was discarded and the pellet washed with ice-cold 70% (v/v) ethanol (0.5 ml), air-dried for 10-15 min and resuspended in 20-40 μl TE buffer which was, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, with or without RNAase at 40 μg/ml. (Sambrook *et al.*, 1989).

2.2.5.2 Midscale preparation

LB media (50 ml) was inoculated with *E. coli* containing the plasmid of interest and amplified overnight. Overnight culture (50 ml) was centrifuged at 300 g for 15 min at room temperature. The supernatant was discarded and the
pellets resuspended in TGE buffer (5 ml) with lysozyme, at a final concentration of 4 mg/ml. This was incubated at room temperature for 15 min. Alkaline SDS (10 ml) was added, mixed and incubated at room temperature for 5 min. Potassium acetate, 3 M, pH 4.8 (7.5 ml) was added. The mixture was incubated at room temperature for 5 min, then centrifuged at 300 g for 15 min at room temperature. An equal volume of propan-2-ol was added to the decanted supernatant, this was mixed by inversion and incubated at room temperature for 15 min. After centrifugation at 300 g for 15 min at room temperature, the supernatant was discarded and the pellet resuspended in TE buffer (5 ml) containing RNAase A at a final concentration of 25 μg/ml. The solution was incubated at 37°C for 30-60 min. Sodium chloride solution, 5 M (5 ml) was added and the solution extracted with an equal volume of phenol/chloroform solution. After centrifugation at 300 g for 5 min at room temperature, the aqueous (upper) layer was removed and added to an equal volume of propan-2-ol. This was incubated at room temperature for 30 min followed by centrifugation at 300 g for 15 min at 4°C, the supernatant was discarded and the pellet washed with ice cold 70% (v/v) ethanol, dried and dissolved in TE buffer (0.5 ml).

2.2.5.3 Large scale preparation

Large scale plasmid preparations were prepared either by ethidium bromide/caesium chloride centrifugation or with the QIAGEN™ plasmid maxi-kit.

Ethidium Bromide/Caesium Chloride Centrifugation: For this procedure, *E. coli* containing the plasmid of interest were amplified overnight in TB media (400 ml) + ampicillin (100 μg/ml) at 37°C while shaken at 300 rpm. The preparation of TB media was as follows, 12 g/l tryptone, 24 g/l yeast extract broth and 4 ml/l glycerol were mixed in water and the solution autoclaved. When cool it was
supplemented with 100 ml/l of phosphate buffer, which consisted of 0.17 mM KH\textsubscript{2}PO\textsubscript{4} and 0.72 mM K\textsubscript{2}HPO\textsubscript{4}.

The overnight culture was transferred into 2 ice-cold centrifuge tubes and incubated on ice for 10 min and centrifuged at 20000 g for 10 min at 4°C. The supernatant was discarded and each pellet resuspended, by pipetting in ice-cold TGE buffer (2.5 ml) and transferred to 2 fresh, pre-cooled centrifuge tubes. TGE buffer (2.5 ml) and lysozyme (to a final concentration of 10 mg/ml) was added to each tube. They were incubated at room temperature for 5 min. Freshly prepared alkaline SDS (10 ml) was added and mixed by inversion. Following a 10 min incubation on ice, potassium acetate, 3 M, pH 4.8 (6 ml) was added and the tubes mixed by inversion. After a further 10 min incubation on ice, the tubes were centrifuged at 30000 g for 35 min at 4°C. The supernatants were transferred into 2 fresh centrifuge tubes and propan-2-ol (12.5 ml) added and mixed. The tubes were incubated at room temperature for 15-30 min, then centrifuged at 10000 g for 20 min at 20°C. The supernatants were discarded, the DNA pellets washed with ice-cold 70\% (v/v) ethanol and air-dried. Each pellet was dissolved in TE buffer, pH 8.0 (4 ml) and caesium chloride (4 g) was added and dissolved, followed by ethidium bromide solution (200 µl of a 10 mg/ml stock). The tubes were centrifuged at 10000 g for 20 min at 4°C. The supernatants were transferred to 2 Beckman Quickseal centrifuge tubes, using a disposable 2 ml syringe fitted with a 21 gauge needle. Before being heat-sealed, these were filled and balanced with a solution of caesium chloride (2 g) in TE buffer (2 ml). The tubes were centrifuged at 400000 g for 16 h at 25°C in a VTi-65 rotor.

The plasmid DNA band was removed with a disposable 1 ml syringe fitted with a 21 gauge needle and placed in a microfuge tube. Ethidium bromide (80 µl of a 10 mg/ml stock) was added and mixed. The solution was transferred to fresh Beckman Quickseal centrifuge tubes as before. These were filled and balanced with a solution of caesium chloride (8 g) in TE buffer (10 ml). They
were centrifuged for 400000 g for 16 h at 25°C in a VTi-65 rotor. After centrifugation, the plasmid DNA band was removed as above and placed in a fresh microfuge tube. (Sambrook et al., 1989).

**Removal of ethidium bromide:** An equal volume of butan-1-ol was added to the DNA-ethidium bromide solution, mixed and centrifuged at 12000 g for 30 sec at room temperature. The upper layer of butan-1-ol and ethidium bromide was removed and discarded. The procedure was repeated until all the pink colouration had left the DNA solution; this was approximately 4-5 times. (Sambrook et al., 1989).

**Removal of caesium chloride:** The DNA solution was dialysed against TE buffer pH 8.0 (1.5 l) for 48 h, with frequent changes of buffer. (Sambrook et al., 1989).

**QIAGEN™ Plasmid Maxi-Kit:** For this procedure *E. coli* containing the plasmid of interest were amplified overnight in LB media (500 ml) + ampicillin (100 µg/ml) at 37°C while shaken at 300 rpm. The overnight culture was transferred into centrifuge tubes and centrifuged at 20000 g for 15 min at 4°C. The supernatant was discarded. The pellet was resuspended in P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 100 µg/ml RNAase A) (10 ml). P2 buffer (200 mM NaOH and 1% (v/v) SDS) (10 ml) was added and mixed. The solution was incubated for 5 min at room temperature. Chilled P3 buffer (2.55 M potassium acetate, pH 4.8) (10 ml) was added and mixed by inversion, followed by 20 min incubation on ice and centrifugation at 15000 g for 30 min at 4°C. The supernatant was promptly removed to a fresh tube. If the solution was turbid, it was recentrifuged at 15000 g for 10 min at 4 °C so that a clear, particle-free lysate was obtained.

The QIAGEN™ 500 tip was equilibrated with QBT buffer (50 mM 3-[N-Morpholino]propane-sulphonic acid (MOPS), pH 7.0, 750 mM NaCl, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100) (10 ml). After the column had drained, the clear supernatant was applied to the column and allowed to flow under gravity.
The column was washed with QC buffer (50 mM MOPS, pH 7.0, 1 M NaCl, 15% (v/v) ethanol) (2 x 30 ml) and the DNA eluted with QF buffer (50 mM MOPS, pH 8.2, 1.25 M NaCl, 15% (v/v) ethanol) (15 ml). Propan-2-ol (0.7 vol) was added to precipitate the DNA and the solution was centrifuged at 15000 g for 30 min at 4°C. The pellet of DNA was carefully washed with ice-cold 70% (v/v) ethanol (15 ml), air-dried and dissolved in TE buffer (0.5-1.0 ml).

2.2.6 Spectrophotometric Quantification of DNA

The concentration and the purity of the DNA solution was determined by measuring the optical density at \( \lambda = 260 \text{ nm} \) and \( \lambda = 280 \text{ nm} \). At \( \lambda = 260 \text{ nm} \) an OD = 1 is approximately equivalent to 50 \( \mu \text{g/ml} \) for double-stranded (ds) DNA, 40 \( \mu \text{g/ml} \) for single-stranded (ss) DNA and 20 \( \mu \text{g/ml} \) for oligonucleotides. The ratio of the optical densities, \( \lambda_{260 \text{ nm}} / \lambda_{280 \text{ nm}} \) determines the purity of the DNA. Pure solutions have a ratio of 1.8-2.0 (Sambrook et al., 1989).

2.2.7 Flat-Bed Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out using the Flowgen minigel apparatus MH 1070. A stock solution of 10 x Tris-borate buffer (TBE) (0.9 M Tris base, 0.9 M boric acid and 40 mM EDTA) was diluted 1:10 with water and agarose (0.9% w/v) was added. The agarose was dissolved by autoclaving for 30 min and the solution then stored at 60°C.

Agarose/ethidium bromide gels were prepared by the addition of ethidium bromide (5 \( \mu \text{l} \) of 10 mg/ml stock) to pre-cooled (45-50°C) agarose solution (40 ml). This was poured into the minigel apparatus with the aluminium casting gates and well comb present. The gel was left to polymerise for 30 min at room temperature before the casting gates and comb were removed and 1 x TBE buffer poured over to a depth of 2-3 mm.

Samples for analysis by agarose gel electrophoresis were prepared by mixing the DNA solution (~100 ng) with 6 x gel-loading buffer (0.25% (w/v)
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bromophenol blue, 30% (v/v) glycerol and 60 mM EDTA) (2 µl) and water added to a final volume of 12 µl. Electrophoresis was carried out at 50 V for 30-60 min at room temperature. The products were visualised with a Flowgen UV Transilluminator (TF-10M) at λ = 312 nm. (Sambrook et al., 1989). Photographs were taken under UV illumination using a Polaroid DS34 instant camera fitted with a Wratten 22A filter and Polaroid 665 positive/negative instant film. Exposures were for 30 sec at f11, followed by development for 30 sec. The negatives were cleared in 18% (w/v) sodium thiosulphate solution for 60 sec, followed by washing for 5 min in water.

2.2.8 Restriction Enzyme Digestion

DNA (0.5-1.0 µg) was mixed with sterile water to a volume of 17 µl. The appropriate 10 x restriction buffer (2 µl), as supplied by the manufacturer, was added and mixed and kept on ice. The restriction enzyme (1 µl, 5-10 units) was added, mixed and briefly centrifuged. The digestion reaction was incubated at 37°C for 1-2 h and terminated by placing on ice or by heating at 70°C for 10 min. An aliquot was analysed by flat-bed agarose gel electrophoresis as described in 2.2.7.

For increased amounts of DNA the volumes were scaled up as appropriate.

2.2.9 Purification of DNA

Purification of DNA was routinely carried out to remove enzymes or other proteins present before further enzymatic reactions. This was done by either Magic™ DNA clean-up resin or by QIAEX DNA resin.

Magic™ DNA clean-up resin: The Magic™ DNA clean-up resin (1 ml) was added to the DNA sample (50-500 µl) in a microfuge tube and mixed well. A syringe barrel, 2 ml, was connected to the supplied mini-column. The DNA-
resin slurry was pipetted into the syringe barrel and gently pushed into the mini-column with the syringe plunger. The resin within the column was washed with 80% (v/v) propan-2-ol (2 ml) and the eluent discarded. The column was placed in a microfuge tube and centrifuged at 12000 g for 20 sec at room temperature to remove the propan-2-ol residue. After standing at room temperature for 10-15 min, prewarmed 65-70°C TE buffer pH 7.5 (40 μl) was applied to the mini-column and incubated for 1 min at room temperature. The DNA was then eluted from the mini-column by centrifugation at 12000 g for 20 sec at room temperature. An aliquot was analysed by flat-bed agarose gel electrophoresis as described in 2.2.7.

**QIAEX™ DNA resin:** The DNA sample (up to 300 μl) was placed in a microfuge tube and buffer QX2 (10 mM Tris-HCl, pH 7.0, 8 M NaClO₄) (3 vol) was added. The QIAEX suspension was briefly vortexed and an aliquot added to the DNA solution (10 μl for every 5 μg of DNA). The sample was incubated at room temperature for 10 min, with mixing every 2 min to keep the resin in suspension. After 10 min, the sample was centrifuged at 12000 g for 30 sec at room temperature and the supernatant discarded. The pellet was washed twice with QX3 buffer (10 mM Tris-HCl, pH 7.5, 70% (v/v) ethanol, 100 mM NaClO₄) (500 μl) and air-dried for 10-15 min. TE buffer (20 μl) was applied, the pellet resuspended and incubated at room temperature for 5 min with mixing every 2 min. The sample was centrifuged at 12000 g for 30 sec at room temperature and the supernatant transferred to a fresh microfuge tube. A second elution was carried out using 10 μl of TE buffer and the 2 eluants combined. An aliquot was analysed by flat-bed agarose gel electrophoresis as described in 2.2.7.

2.2.10 Isolation of DNA Fragments

Digested DNA was analysed by 0.9% (w/v) flat-bed agarose gel electrophoresis as described in 2.2.7. except that the agarose gel was cast with
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a 50 µl well comb. The samples were mixed with 6 x gel-loading buffer and 30-50 µl were loaded per well and electrophoresed for 60 min at 50 V. The DNA bands were visualized under UV illumination. The desired bands were excised with a sterile scalpel blade and placed in microfuge tubes.

2.2.11 DNA Extraction from Agarose Gel

This was carried out using the QIAEX resin according to the manufacturers recommendation.

The gel slice was solubilized in QX1 buffer (10 mM Tris-HCl, pH 7.0, 3 M NaI, 4 M NaClO₄, 10 mM sodium thiosulphate) (300 µl per 100 mg of gel) and 0.1 vol mannitol (1 M). An aliquot of resuspended QIAEX resin (10 µl per 5 µg DNA) was added and the sample incubated at 50°C for 10 min, with brief vortexing every 2 min. The sample was centrifuged at 12000 g for 30 sec at room temperature and the supernatant discarded. The pellet of DNA-resin was washed twice with QX2 buffer (10 mM Tris-HCl, pH 7.0, 8 M NaClO₄) (500 µl) to remove agarose contaminants. It was washed twice with QX3 buffer (10 mM Tris-HCl, pH 7.5, 70% (v/v) ethanol, 100 mM NaClO₄) (500 µl) to remove salt contaminants. The pellet was air-dried for 10-15 min and then resuspended in TE buffer pH 8.0 (20 µl). The suspension was incubated at room temperature for 5 min, with brief vortexing every 2 min. The sample was centrifuged at 12000 g for 30 sec at room temperature. The supernatant was transferred to a clean microfuge tube. If necessary, a second elution was carried out using 10 µl of TE buffer pH 8.0; this increased the yield of DNA by 10-15%.

2.2.12 Dephosphorylation of Linearized DNA

Dephosphorylation was carried out to remove the 3'-phosphate groups from linearized, compatible-ended DNA to prevent self-ligation. The digested, purified DNA was mixed with 0.1 vol 10 x calf intestinal phosphatase (CIP) dephosphorylation buffer (100 mM Tris-HCl, pH 8.3, 10 mM MgCl₂, 10 mM
ZnCl₂). CIP (2 units, 2 μl) was added and the reaction incubated at 37°C for 1-2 h. Volumes were calculated so that the enzyme volume was less than 10% of the total. The reaction was stopped by incubation at 70°C for 10 min. The DNA was purified by one of the methods in 2.2.9. An aliquot was analysed by flat-bed agarose gel electrophoresis as described in 2.2.7.

2.2.13 Ligation of DNA Fragments

Ligation of DNA fragments was carried out on digested, isolated and purified DNA. The vector DNA (100-150 ng/μl) was mixed with the cDNA of interest in molar ratios of 1:1, 1:2 and 1:3. Sterile water was added to a total volume of 10 μl. Ligase mix was prepared. This consisted of T4 DNA ligase (2 units, 2 μl), 10 x buffer (660 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 10 mM dithiothreitol, 10 mM adenosine triphosphate (ATP)) (2 μl) and water (6 μl). The ligase mix (10 μl) was added to the DNA solution. After gentle mixing the reactions were incubated for either 24 h at 4°C or 16 h at 16°C. An aliquot (5 μl) was then removed and used to transform competent E. coli as described in 2.2.4.

2.2.14 Preparation of DMEM/F12 Media for the Culture of HEK 293 Cells

One bottle of powdered DMEM/F12 1:1 mix, which contained l-glutamine and 15 mM HEPES but did not contain sodium bicarbonate, was mixed in sterile water, 800 ml. To this was added sterile foetal calf serum (FCS) (100 ml), penicillin-streptomycin solution (20 ml), to final concentrations of 500 IU/ml penicillin and 500 μg/ml streptomycin, and 7.5% (w/v) sodium bicarbonate (40 ml), final concentration of 3 g/l. The total volume was made up to 1 l with sterile water and the pH adjusted to pH 7.65 with NaOH (2 M). The media was filtered using a 0.2 μm Sartorius Sartolab-V150 filter unit and stored at 4°C.
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Liquid DMEM/F12 media which did not contain l-glutamine was used for the culturing of HEK 293 cells during transfection. This media which did contain 15 mM HEPES and some sodium bicarbonate was prepared as above. However, the amount of 7.5% (w/v) sodium bicarbonate added was reduced to 24 ml due to the presence of sodium bicarbonate within the liquid DMEM/F12 media. The final concentration of sodium bicarbonate (3 g/l) remained the same in both types of media.

2.2.15 Culturing of HEK 293 cells

HEK 293 cells were normally grown in 250 ml Greiner Tissue Culture flasks at 37°C in 5% carbon dioxide in the DMEM/F12 media which contained l-glutamine. Every 2-3 days they were subcultured. This was carried out as follows; the media was removed, the cells were washed with Hanks buffered salt solution (4 ml), this was removed and the cells trypsinized from the flask with 1 x trypsin-EDTA solution (porcine trypsin 0.5 g/l and EDTA 0.2 g/l) (2 ml). Trypsinization was carried out at 37°C for 60 sec. The addition of fresh DMEM/F12 media (10 ml) inhibited further trypsinization. The cells were separated by gentle pipetting. The suspension (2 ml) was placed in a fresh flask, DMEM/F12 media was added (12 ml) and the flask returned to the incubator.

2.2.16 Liquid Nitrogen Storage of HEK 293 Cells

For long term storage, HEK 293 cell stocks were kept in liquid nitrogen. These stocks were prepared from cells cultured at a lower density in 600 ml Greiner Tissue Culture flasks. The cells were dissociated using trypsin-EDTA (4 ml) as described above. Trypsinization was terminated by the addition of DMEM/F12 media (20 ml), as above. The cells were pelleted by centrifugation at 200 g for 5 min at 4°C. The pellet was resuspended in DMEM/F12 media (4.8 ml), mixed with a 1:1 solution (1.2 ml) of FCS and dimethyl sulphoxide (DMSO). The suspension was immediately divided into 3 cryogenic vials. These
were stored for 24 h at -80°C in a polystyrene box and then transferred to liquid nitrogen.

When a new culture was required, a single cryogenic vial of HEK 293 cells was thawed at 37°C in a waterbath. The cells were centrifuged at 200 g for 5 min at 4°C and the supernatant removed. The pellet was resuspended in DMEM/F12 media which contained l-glutamine (15 ml). The suspension was pipetted into a fresh flask and placed in the incubator, where the cells were maintained as described in 2.2.15.

2.2.17 Transfection of HEK 293 Cells

Transfection of HEK 293 cells was carried out by the calcium phosphate precipitation method of Gorman et al., (1990). Stock solutions were prepared prior to transfection. These were:

i) 1/10 TE buffer (TE buffer, pH 8.0 diluted 1:10 in sterile water)

ii) 2 X HEPES buffered saline (HBS) (50 mM HEPES, pH 7.12, 280 mM NaCl, 1 mM Na$_2$HPO$_4$)

iii) 2.5 M calcium chloride

iv) Phosphate buffered saline (PBS) (4 mM Na$_2$HPO$_4$, 1.7 mM KH$_2$PO$_4$, pH 7.4, 137 mM NaCl, 107 mM KCl)

v) 15% (v/v) glycerol in PBS

Cells were subcultured to a density of 3-4 x 10$^6$ cells per flask 24 h prior to transfection. On the day of the procedure and 3.5 h before transfection, the media was removed from the cells and replaced with fresh l-glutamine-free DMEM/F12 media (10 ml/flask). Importantly, the CO$_2$ level in the incubator was increased from 5% to 7.5%, to give the media a pH of 7.3-7.4 during the transfection. At 30 min prior to transfection, the precipitating solutions were prepared:

Tube A; 440 µl 1/10 TE buffer, pH 8.0 (or 430 µl)

10 µl DNA (1 µg/µl) (or 20 µl)
Calcium chloride (2.5 M, 50 μl) was added to Tube A and mixed well. Tube A was slowly added dropwise to Tube B, over 1 min. After this, the solutions were mixed by pipetting up and down x 10 to aid calcium phosphate precipitate formation. The precipitated solution was added slowly, drop by drop, to the media over the HEK 293 cells. The flask was gently swirled to mix the precipitated solution evenly and the flask returned to the incubator.

After 3.5-4 h the media was aspirated from the cells by pipette and 15% (v/v) glycerol in PBS (1.5 ml) was spread gently over the cells. After 30 s the glycerol solution was removed and the cells rinsed with a small volume (~2-4 ml) of l-glutamine-free DMEM/F12 media. Fresh l-glutamine-free DMEM/F12 media (10 ml) was then added and the cells returned to the incubator for 40 h. The cells were harvested as below (2.2.19) and analysed for the expression of the desired protein.

2.2.18 Assessing the Efficiency of Transfection

The efficiency of transfection was routinely monitored using the plasmid pSV-β-galactosidase and the chromogenic substrate x-gal as described by Lim and Chae (1989). Transfected HEK 293 cells express the enzyme β-galactosidase, and upon incubation with the x-gal substrate these cells appear blue due to the utilization of the chromogenic substrate.

The staining procedure was carried out 24-48 h after the HEK 293 cell transfection. The following stock solutions were prepared prior to the assay;

i) Phosphate buffered saline (PBS) (1.4 mM KH$_2$PO$_4$, pH 7.1, 4.3 mM Na$_2$HPO$_4$, 137 mM NaCl, 2.7 mM KCl)

ii) 0.1 M Sodium phosphate buffer (60 mM Na$_2$HPO$_4$, pH 7.0, 40 mM NaH$_2$PO$_4$)

iii) Gluteraldehyde solution (0.1 M sodium phosphate buffer, pH 7.0, 1 mM MgCl$_2$, 1% (v/v) gluteraldehyde)
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iv) X-gal solution (10 mM sodium phosphate buffer, pH 7.0, 1 mM MgCl₂, 150 mM NaCl, 3.3 mM K₄Fe[CN]₆.3H₂O, 3.3 mM K₃Fe[CN]₆, 0.2% (w/v) x-gal)

The HEK 293 cells were transfected with pSV-β-galactosidase (10 µl) as described 2.2.17. After the transfection (24-48 h), the media was aspirated and the cells were washed with PBS buffer, pH 7.1 (10 ml x 5). Gluteraldehyde solution (15 ml) was added gently to the cells and the flask incubated for 15 min at 4°C. The gluteraldehyde solution was removed and the x-gal solution (2 ml) gently swirled over the cells. The cells were incubated for 30-60 min at 37°C with gentle shaking. After incubation, the x-gal solution was removed and the cells fixed with 70% (v/v) glycerol. Transfected (blue) cells were identified under a light microscope at magnification x 200.

2.2.19 Harvesting of the Transfected HEK 293 Cells

Cells were scraped into the culture media within the flask and placed in centrifugation tubes, flasks were rinsed with Tris-acetate buffer, pH 7.4 (50 mM Tris-acetate, pH 7.4, 5 mM EDTA, 5 mM EGTA) which was added to the centrifugation tubes. The cells were pelleted by centrifugation at 500 g for 5 min at 4°C. The supernatant was discarded and the pellets homogenized in ice-cold Tris-acetate buffer 50 mM, pH 7.4, using a Wheaton Dounce, glass-glass tissue grinder (12 strokes). The homogenate was centrifuged at 20000 g for 30 min at 4°C. The supernatant was discarded and the pellet resuspended by homogenization in Tris-acetate buffer, 50 mM, pH 7.4 to a final concentration of approximately 1 mg protein/ml. If the protein was to be used for a radioligand binding assay involving Ca²⁺, Mg²⁺ or Zn²⁺ ions, the final resuspension was carried out in Tris-acetate buffer, 50 mM, pH 7.4 without chelators. The cell suspension was stored at -20°C, if not used immediately (Chazot et al., 1992).
2.2.20 Membrane Preparation of Native Receptors

Cerebella tissue was dissected from adult mice, Balb/c strain. The tissue was homogenized in ice-cold Tris-HCl buffer pH 7.4 (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 200 mM sucrose, 0.02% (w/v) NaN₃) (9 vol w/v) to which was added protease inhibitors, trypsin II-S, trypsin II-O, benzamide and bacitracin, all to a final concentration of 10 µg/ml and phenylmethyl sulphonyl fluoride (PMSF) to a final concentration of 0.5 µg/ml. A Potter glass-teflon homogenizer (12 strokes) was used to homogenise the tissue. The homogenate was centrifuged at 800 g for 10 min at 4°C. The supernatant was stored on ice. The pellet was rehomogenized in ice-cold Tris-HCl buffer, pH 7.4 (4.5 vol (w/v), 6 strokes) and recentrifuged at 800 g for 10 min at 4°C. The first and second supernatants were combined and centrifuged at 126000 g for 60 min at 4°C. The supernatant was discarded. The pellet was homogenised in Tris-HCl buffer, 50 mM, pH 7.4. The homogenate was then; snap-frozen in liquid nitrogen, thawed, rehomogenized and centrifuged at 126000 g for 30 min at 4°C. This freeze-thaw cycle was repeated x 5. The final pellet was resuspended in Tris-acetate buffer, 50 mM, pH 7.4, 2 vol (w/v), aliquotted and stored at -20°C (Chazot et al., 1992).

2.2.21 Determination of Protein Concentration

The protein concentration was determined by the method of Lowry et al. (1951). Stock reagents were prepared prior to the assay.

Reagent A; 2% (w/v) sodium carbonate
0.1 M NaOH
5% (w/v) SDS

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

Reagent C; 1% (w/v) copper sulphate

To the required volume of Reagent A were added Reagents B and C to a final concentration of 0.02% and 0.01% respectively. This solution (1 ml) was added to the protein sample (50 µl), vortexed and incubated for 10 min at room
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temperature. Folin-Ciocalteau phenol reagent (1 M, 100 μl) was added and mixed. The assay was incubated for 30 min at room temperature. The OD at λ = 750 nm was measured. A calibration curve was determined with bovine serum albumin (BSA) as the standard protein. All samples were assayed in triplicate.

2.2.22 Radioligand Binding Assays

The methodology for the [³H]MK801 radioligand binding assays for native brain membranes and transfected HEK 293 cell membranes was a polyethyleneimine filtration assay. Protein (~100 μg), prepared as described in 2.2.19 and 2.2.20, was incubated with L-glutamate (10 μM) and [³H]MK801 in Tris-acetate buffer, 50 mM, pH 7.4 to a total volume of 200 μl for 3 h at room temperature. Non-specific binding was determined in the presence of (+)MK801 (10 μM). Total and non-specific binding samples were always assayed in triplicate. Samples were filtered, using 1% (v/v) polyethyleneimine pre-soaked Whatman GF/B filters, on a Brandel M-24R cell harvester and washed x 3 with 10 mM potassium dihydrogen phosphate buffer, pH 7.4. The filters were placed in minivials, Optiphase 'safe' scintillation fluid (3 ml) added and incubated for 16 h at room temperature. The vials were vigorously vortexed before counting. Counting was carried out in a Beckman LS 5000CE scintillation spectrophotometer with a counting time of 4 min per sample.

For saturation binding analysis, protein solution was incubated with a range of [³H]MK801 concentrations (1-70 nM). For single point analysis and competition experiments, the [³H]MK801 concentration used was dependent upon the receptor being studied. For native cerebella receptors and NR1α/NR2A/NR2C 1:3:3 and 1:10:3 recombinant receptors, the [³H]MK801 concentration was 20 nM. For the recombinant receptors NR1α/NR2C 1:10 and NR1α/NR2A/NR2C 1:3:10, 50 nM [³H]MK801 was used. Saturation binding and drug displacement curves were analysed by Inplot computer program.
2.2.23 SDS-Polyacrylamide Gel Electrophoresis

Resolving Gel Preparation: Water (25 ml) was mixed with 1x electrophoresis buffer, pH 8.8 (50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS), 30% (w/v) acrylamide stock (20 ml) and TEMED (30 μl). This solution was degassed for 20 min. Following degassing, 10% (w/v) ammonium persulphate (APS) (300 μl) was added. The gel solution was immediately poured into a Biotech Instruments Ltd Gel Caster for 10 gels, using gel plates of 10 x 8 cm and spacers of 1 mm width. Water-saturated butan-1-ol solution (100 μl) was poured over the top of each gel to provide a 'smooth' top to the gels, which were then left to polymerise for 60 min at room temperature. The water-saturated butan-1-ol solution was rinsed off with fresh water and the gels individually wrapped and stored at 4°C. The final acrylamide concentration of the resolving gels was 7%.

Stacking Gel Preparation: Stacking gel buffer (0.5 M Tris-glycine, pH 6.8, 8 mM EDTA and 0.4% (w/v) SDS) (1 ml) was mixed with water (2.3 ml), 30% (w/v) acrylamide stock (650 μl) and TEMED (2.5 μl). This solution was degassed for 10 min. After degassing, 10% (w/v) APS (80 μl) was added. The gel solution was poured immediately, the comb inserted and left to set (~2-10 min). The final acrylamide concentration of the stacking gel was 5%.

Sample Preparation: The protein samples (50 μg, prepared as in 2.2.19 and 2.2.20) were centrifuged at 12000 g for 5 min at 4°C and the supernatant carefully removed. The protein pellet was resuspended in sample buffer (30 mM NaH₂PO₄, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS) (5 μl), 100 mM dithriothreitol (DTT) (1.5 μl) and water to a final volume of 15 μl by vortexing. The sample was boiled for 3 min, then centrifuged at 12000 g for 30 sec at room temperature.

Conditions of SDS-PAGE electrophoresis: Electrophoresis was carried out using the Hoefer Mighty Small II vertical slab unit SE250. Samples were run into the stacking gel at constant current of 10 mA for 20 min in 1x...
electrophoresis buffer pH 8.8 (50 mM Tris, 384 mM glycine, 1.8 mM EDTA and 0.1% (w/v) SDS). The resolving gel was a prepoured 7% (w/v) acrylamide slab gel. Electrophoresis was carried out at a constant current of 15 mA for ~2 h until the bromophenol blue band had reached the bottom of the gel, or alternatively, until the 30.5 kDa molecular weight standard was at the bottom of the gel. This allowed a better resolution of the NMDA subunit proteins of interest.

2.2.24 Immunoblotting of SDS-PAGE Gels

Transferring of Proteins to Nitrocellulose: Within a transfer cassette a 'sandwich' was constructed. This consisted of a sheet of sponge, 2 sheets of blotting paper, a sheet of nitrocellulose, the protein gel, a further 2 sheets of blotting paper and a further sheet of sponge. All components of the transfer 'sandwich' were presoaked in transfer buffer which is 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.4. Moreover, as each new layer was added, it was pressed so that any trapped air-bubbles were removed. The proteins were transferred at 50 V (constant) for 2.5 h in a Hoefer TE series Transphor Electrophoresis unit, containing transfer buffer (2 1).

Blocking of Nitrocellulose Membrane: Following transfer of the proteins to the nitrocellulose membrane and prior to incubation with the primary antibodies, the non-specific, antibody-binding sites were blocked by immersion in a solution composed of, 5% (w/v) milk powder, 0.02% (v/v) Tween-20 in Tris buffered saline (TBS), pH 7.4 (50 ml). Blocking was carried out overnight at 4°C with gentle shaking.

Incubation with Primary Antibody: Following the overnight blocking of the non-specific binding sites, the nitrocellulose membrane was rinsed with TBS, pH 7.4 and incubated with the appropriate primary antibody in a solution of 2.5% (w/v) milk powder in TBS, pH 7.4. The membrane was incubated for 60 min at 37°C with gentle shaking. The antibodies used were anti-NR1-(911-
920), anti-Cys-NR2C-(1-14), anti-NR2C-(1208-1218) and anti-NR2A-(1432-1442). These antibodies were characterized by Dr. P. L. Chazot.

Washing of Membrane: Following the incubation, the primary antibody solution was removed and the membrane was washed 4x in a solution of 2.5% (w/v) milk powder and 0.2% (v/v) Tween-20 in TBS, pH 7.4 (20 ml) for 10 min at 37°C with gentle shaking. This removed all the unbound primary antibody.

Incubation with Secondary Antibody: After the washing steps, the nitrocellulose membrane was incubated with the secondary antibody. This was horseradish peroxidase labelled anti-rabbit antibody at a dilution of 1/1500 (v/v) in a solution of 2.5% (w/v) milk powder in TBS, pH 7.4. The membrane was incubated for 60 min at 37°C with gentle shaking. This antibody specifically bound to the primary antibody.

Washing and Rinsing of Membrane: Following the incubation, the secondary antibody solution was removed and the membrane was washed 4x in a solution of 2.5% (w/v) milk powder and 0.2% (v/v) Tween-20 in TBS, pH 7.4 (20 ml) for 10 min at 37°C with gentle shaking. This removed all the unbound secondary antibody. The membrane was then rinsed 2x in TBS, pH 7.4 (50 ml).

Detection by ECL: The protocol was carried out as recommended by the manufacturer. Detection solution 1 and detection solution 2 were mixed with a 1:1 (v/v) ratio. The blot was drained of excess TBS buffer and incubated in the detection solution for precisely 60 sec at room temperature. After removal, the excess reagent was drained and the blot wrapped in cling-film. The blot was placed - protein side up - in a film cassette and exposed to Hyperfilm™, ECL film. Exposures were from 30 sec to 5 min. The film was developed in Kodak D-19 Developer and fixed in Kodak Unifix.

Quantification of Western Blots: Developed Hyperfilm™ exposures of Western blots were scanned on a Molecular Dynamics Personal Densitometer and the band intensities analysed using ImageQuant™ software. A range of protein concentrations (1-50 μg) was firstly run and probed, and the Hyperfilm™
scanned. An appropriate protein concentration was chosen which gave a reading in the linear range of the calibration curve, this concentration was used for Western blots which would be quantified. Only bands probed by the same primary antibody and within the same gel and exposure were quantified to avoid variation due to the Western blotting procedure and Hyperfilm™ development.
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RESULTS

2.3.1 The Pharmacology of Native Cerebellar NMDA Receptors

Previous studies have shown localised, high levels of expression of the NR2C mRNA in the cerebellum (Monyer et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993). In order to correlate the properties of the cloned receptor, a detailed study was made of [$^3$H]MK801 binding to the native cerebellar receptor of adult mice. Radioligand binding was carried out as described in 2.2.22. A typical saturation curve for [$^3$H]MK801 binding on native cerebellar receptors is shown in Figure 2.3.1. Binding was saturable to a single high affinity site with a $K_D$ of 22 ± 9 nM (Table 2.3.1). Displacement assays were also carried out using a single concentration of [$^3$H]MK801 and increasing concentrations of unlabelled (+)MK801. These were best fit to a single site, with a displacement constant of $K_D = 26 ± 4$ nM (n = 3). Further pharmacological investigation was carried out on the native mouse cerebellar receptor, with ketamine, TCP, Mg$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$, the results of which are summarised in Table 2.3.1. Typical displacement curves are shown in Figure 2.3.2. All the displacement curves were fitted to a one site model.

2.3.2 Subcloning of the NR2C cDNA into the pCIS Vector

The pBKSII+NR2C construct was provided by Dr M. Mishina. The restriction map is shown in Figure 2.3.3. The subcloning of the NR2C clone into the pCIS vector was necessary for efficient expression in the HEK 293 cells. The NR2C cDNA was excised from the pBKSII+ plasmid (Kutsuwada et al., 1992) by EcoRI digestion and subcloned into the mammalian expression vector, pCIS (Figure 2.3.5). This generated the construct pCISNR2C, as shown in Figure 2.3.6. The strategy was chosen due to the presence of untranslated regions at the 5' and 3' end of the NR2C cDNA, as these regions were not sequenced there was no information as to the restriction sites which might be present. The subcloning
was sticky-ended but non-directional, however the presence of an EcoRI site in the untranslated regions would not cause problems.

The full length NR2C cDNA, a reading frame encoding 1220 amino acids, with the 5' and 3' untranslated regions of approximately 200 bp and 500 bp respectively, was cut from the multicloning site of the pBKSII+ plasmid (Figure 2.3.4) using restriction enzyme EcoRI. This generated a fragment of 4.4 kb. The pBKSII+ plasmid was destroyed in the same reaction by digestion with Scal, generating fragments of 2.0 kb and 1.0 kb. This reduced the possibility for false recombinants after the ligation reaction. The fragments were separated by agarose gel electrophoresis as described 2.2.10. The 4.4 kb band was excised from the agarose gel and purified as described 2.2.11.

The pCIS plasmid was digested with EcoRI. The DNA was purified and dephosphorylated as described in 2.2.9 and 2.2.12 respectively. After a second purification, the ligation reactions were prepared as described in 2.2.13. INV1α competent cells were transformed with 5 μl of the ligation reactions as described (2.2.4). Bacterial colonies were picked and grown overnight. Small-scale plasmid preparation (2.2.5.1) was carried out and the resulting DNA analysed by agarose gel electrophoresis. Recombinant pCISNR2C DNA was determined by size (9.1 kb). The pCIS (4.7 kb) and pBKSII+NR2C (7.3 kb) were also analysed. The orientation of recombinants was checked by restriction enzyme digestion with Sall. A recombinant of the correct orientation will generate 2 fragments of 5.4 kb and 3.7 kb; a wrong orientation will generate fragments of 8.4 kb and 0.6 kb. The selected recombinant was further analysed by restriction enzyme digestion (Figure 2.3.7). Lanes 2, 3 and 5 show pBKSII+NR2C, pCIS and pCISNR2C respectively. The correct orientation and size of pCISNR2C are shown in lanes 7 and 8. In lane 6 the pCISNR2C is digested with EcoRI demonstrating the excision of the NR2C cDNA.
2.3.3 Transient Expression of Recombinant pCISNR2C in HEK 293 Cells

The efficiency of transfection was determined and routinely monitored by transfection with pSV-β-galactosidase, followed by X-gal staining. Transfected cells appeared blue under phase-contrast microscopy. An example of the determination of transfection efficiency can be seen in Figure 2.3.8. Following the procedure for transfection as described 2.2.17 transfection efficiencies in the range of 20-35% were routinely achieved.

HEK 293 cells were transfected with the NR2C clone alone. Transfected cells were analysed by both immunoblotting with specific anti-NR2C antibodies and [³H]MK801 radioligand binding assays. Transfected HEK 293 cell membrane homogenates were probed with specific anti-NR2C antibodies, anti-Cys-NR2C-(1-14) and anti-NR2C (1208-1218). Both antibodies recognized a single polypeptide with Mᵋ 145000 ± 2500 (Figure 2.3.9).

Following N-deglycosylation of the transfected cell homogenates the immunoreactive band was detected with a decreased size of Mᵋ 133000 ± 2500 (Figure 2.3.9). This corresponds to the value predicted for the mature polypeptide derived from the cDNA sequence (Kutsuwada et al., 1992; Monyer et al., 1992). No [³H]MK801 binding activity was detected in cell homogenates following transfection with the NR2C subunit clone alone.

Untransfected HEK 293 cells showed no immunoreactivity to either of the anti-NR2C antibodies, nor showed any [³H]MK801 radioligand binding activity, results not shown.

2.3.4 Transient Co-expression of Recombinants; pCISNR1a and pCISNR2C

Following the successful expression of the NR2C subunit in HEK 293 cells, co-transfections with pCISNR1a (Figure 2.3.10) and pCISNR2C (Figure 2.3.6) were carried out. Again, expression of NR1a/NR2C heteromeric receptors
was determined by both $[^3]H$MK801 binding and immunoblotting with subunit-specific antibodies.

Immunoblotting of co-transfected cell membranes demonstrated the presence of NR1a and NR2C subunits, as demonstrated in Figure 2.3.11. Co-transfected HEK 293 cell membrane homogenates were probed with anti-NR1a (911-920) and anti-NR2C (1208-1218) antibodies. The anti-NR1a antibody recognised a single polypeptide band with $M_r$ 117000. This value agrees with that previously reported for the N-glycosylated peptide (Chazot et al., 1992). The anti-NR2C antibody recognised two polypeptide bands with $M_r$ 145000 and $M_r$ 133000 respectively. This differed from HEK 293 cells singly transfected with pCISNR2C which demonstrated only the $M_r$ 145000 band. The lower band corresponds to the value predicted for the non-glycosylated mature polypeptide (Monyer et al., 1992; Kutsuwada et al., 1992). Prior incubation of the antibodies with their respective polypeptides, which had been used for immunisation, blocked both signals. This is shown in Figure 2.3.11.

2.3.4.1 Optimization of the Expression of the Recombinant NR1a/NR2C Receptor Population as Measured by $[^3]H$MK801 Binding

It has been previously reported that the maximal expression of NR1a/NR2A heteromeric receptors in HEK 293 cells, as determined by both single point $[^3]H$MK801 binding and immunoblotting, was dependent upon the ratio of the respective DNAs used and on the presence of glutamate antagonists in the cell culture media post-transfection (Cik et al., 1993). Therefore, the initial transfection studies for the co-expression of NR1a and NR2C examined the effect of varying the plasmid ratios of the respective constructs. The ratios used were pCISNR1a:pCISNR2C 4:1, 1:1, 1:4, 1:7, 1:10 and 1:14 respectively. As earlier studies had shown that 10 $\mu$g of total plasmid DNA was optimal for the expression of receptors (Cik et al., 1993), the ratios 4:1, 1:1, 1:4 and 1:7 varied
such that the total plasmid DNA used for transfection was maintained at this level (10 μg). However, for the ratios 1:10 and 1:14 the total amount of DNA used for transfection was increased to 11 μg and 15 μg respectively. Control transfections with pCISNR1a alone and $[^{3}H]$MK801 radioligand binding assays on the expressed homomeric NR1a recombinant receptor were carried out in parallel with the NR1a/NR2C studies. The NR1a/NR2C results were calculated as a percentage of the $[^{3}H]$MK801 radioligand binding to the homomeric NR1a receptor and they are summarized in Table 2.3.2. It can be seen that maximum $[^{3}H]$MK801 binding activity was expressed for the DNA ratio of 1:10 NR1a:NR2C. This gave a 2-fold increase in binding compared to NR1a alone (Table 2.3.2). The DNA ratio of 1:7 NR1a:NR2C gave a similar level of $[^{3}H]$MK801 binding activity to NR1a alone; all the other DNA ratios studied gave significantly less $[^{3}H]$MK801 binding activity than the control, NR1a alone. For all further investigations on the recombinant NR1a/NR2C, receptor the 1:10 ratio of DNAs was therefore maintained.

Prior studies on the recombinant NR1a/NR2A receptor expression had shown a need for antagonist protection (Cik et al., 1993). There was a significant increase in dead and floating cells following transfection as compared to untransfected cells or homomeric NR1a transfections. The presence of AP5 (400 μM) significantly increased the $[^{3}H]$MK801 binding activity (Cik et al., 1993). Although there was no evidence of significant cell death post-transfection for the NR1a/NR2C receptor the level of $[^{3}H]$MK801 binding was low. Therefore, the effect of AP5 (400 μM) in the cell culture media post-transfection was assayed by single point $[^{3}H]$MK801 binding for the NR1a/NR2C 1:10 receptor. The presence of the glutamate antagonist significantly reduced $[^{3}H]$MK801 binding (Table 2.3.2). Therefore, AP5 was omitted from further studies on this cloned receptor subtype.
2.3.4.2 [³H]MK801 Pharmacology of the Recombinant NR1a/NR2C Receptor Population

A full saturation curve for the binding of [³H]MK801 was carried out for the recombinant NR1a/NR2C receptor using the 1:10 DNA ratio. However, the specific binding was not saturable at concentrations of [³H]MK801 up to 70 nM, results not shown. Thus the affinity constant was determined by displacement with unlabelled (+)MK801. Figure 2.3.12 shows a typical curve. The value found was $K_i = 346 \pm 158$ nM (n=3), which compared to a $K_D$ of $22 \pm 9$ nM for native adult cerebellar receptors. The values are shown in Table 2.3.4. Full pharmacological characterization of the recombinant NR1a/NR2C receptors was not possible due to the low signal:noise ratio for [³H]MK801 binding. Typical values for total and non-specific binding to recombinant NR1a/NR2C receptors for different concentrations of [³H]MK801 are shown in Table 2.3.3.

2.3.5 Transient Co-expression of Recombinants; pCISNR1a and pCISNR2A and pCISNR2C

The dissociation constant for the binding of [³H]MK801 to the NR1a/NR2C recombinant receptor was significantly different from that of the native cerebellar NMDA receptor. However, Monyer et al. (1992) showed the presence of the NR2A mRNA in adult cerebellum. Further, Wafford et al. (1993) reported the preferential co-assembly of NR1a, NR2A and NR2C subunits following co-expression in Xenopus oocytes. Therefore triple transfections of HEK 293 cells were carried out with NR1a, NR2A and NR2C cDNAs in mammalian expression vectors (Figures 2.3.10, 2.3.13 and 2.3.6 respectively) as described 2.2.17. The cDNA ratios were again varied to discover the optimal DNA ratio for expression. The subunit DNA ratios used in the triple transfections were 1:3:10, 1:3:3 and 1:10:3 of NR1a:NR2A:NR2C respectively. The receptor populations were initially investigated by full saturation curves and immunoblotting.
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For all the transfections, the presence of all three subunits was determined by immunoblotting, as shown in Figure 2.3.16. Transfected HEK 293 cell membrane homogenates were immunoblotted with anti-NR1a (911-920), anti-NR2A (1432-1442) and anti-NR2C (1208-1218) antibodies. Mature, glycosylated subunit polypeptides of NR1a, NR2A and NR2C of molecular weights, \( M_r \), 117000, \( M_r \) 180000 and \( M_r \) 145000 respectively were detected for each ratio used for the triple transfections. The immunoblots also detected non-N-glycosylated NR1a polypeptide of \( M_r \) 97000.

2.3.5.1 The \[^{3}H\]MK801 Pharmacology of the Recombinant NR1a/NR2A/NR2C Receptor Populations

The dissociation constants for the binding of \[^{3}H\]MK801 to the recombinant NR1a/NR2A/NR2C 1:10:3 and 1:3:3 receptor populations were determined, as summarized in Table 2.3.4. The presence of the NR2A subunit clearly increased the \[^{3}H\]MK801 affinity for the 1:3:3 and 1:10:3 DNA ratio receptor populations as compared to the NR1a/NR2C cloned receptor. However, the 1:3:10 DNA ratio receptor population was non-saturating at \[^{3}H\]MK801 concentrations up to 70 nM. This ratio resulted in a low affinity receptor population similar to that found for NR1a/NR2C recombinant receptor population. Attempts to determine the inhibition constant by (+)MK801 displacement were unsuccessful due to the low signal to noise ratio.

The dissociation constants for \[^{3}H\]MK801 binding to the NR1a/NR2A/NR2C receptors with DNA ratios 1:3:3 and 1:10:3 were not significantly different (Table 2.3.4) and both were best fit by a single site model. Moreover, both were similar to the \( K_d \) for \[^{3}H\]MK801 binding to native adult cerebellar NMDA receptors. However, the \( K_d \) values for the 1:3:3 DNA ratio receptor and the NR1a/NR2A cloned receptor were significantly different (\( p < 0.01 \)); while for the 1:10:3 DNA ratio receptor and the NR1a/NR2A cloned receptor their respective \( K_d \) values were not significantly different.
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From the initial triple transfections, in the absence of AP5, it was noted that the co-expression of NR1a/NR2A and NR2C subunits resulted in significant cell death, due to the presence of round and/or floating cells. Previously it had been observed that co-expression of NR1a/NR2C subunits did not give detectable levels of cell death. The level of cell death for the different DNA ratios was quantified by Dr M. Cik, by the method described in 3.2.11. The results are displayed in Table 2.3.5 (Chazot et al., 1994). Therefore, all triple transfections were carried out in the presence of 400 µM AP5. This glutamate antagonist had previously been found to be effective against cell toxicity in the studies upon expression of recombinant NR1a/NR2A receptors in HEK 293 cells (Cik et al., 1993).

2.3.5.2 Pharmacology of the Recombinant NR1a/NR2A/NR2C Receptor Populations

Following the determination of the dissociation constants for the cloned receptor populations NR1a/NR2a/NR2C of cDNA ratios 1:10:3 and 1:3:3, further pharmacological investigation of these receptor populations was performed. However, the low signal to noise ratio of the NR1a/NR2A/NR2C 1:3:10 receptor population which prevented the determination of the $K_d$ for $[^3]H$MK801 binding, also prevented further pharmacological investigation of this receptor population. The inhibition constants of a series of ligands; TCP, ketamine, Mg$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$, were determined in displacement studies as described in 2.2.22, these ligands interact at different sites within the NMDA receptor channel. The results are summarized in Table 2.3.6.

All the displacement curves were fitted to a single site model. No curves were significantly better fitted to a two site model. Of the five compounds tested only ketamine and Ca$^{2+}$ gave significantly different results for the triply transfected cells with DNA ratios 1:3:3 and 1:10:3. The NR1a/NR2A/NR2C 1:3:3 receptor population had a 2-3 fold lower affinity for ketamine, and a 3-4
fold higher affinity for Ca\textsuperscript{2+}, as compared to the NR1a/NR2A/NR2C 1:10:3 receptor population. Interestingly, the NR1a/NR2A/NR2C 1:10:3 receptor population had a similar $K_i$ for ketamine as the NR1a/NR2A 1:3 receptor. However, the affinity for TCP for both NR1a/NR2A/NR2C receptor populations was similar and over 25-fold lower than for the NR1a/NR2A receptor. All three receptor populations (NR1a/NR2A/NR2C 1:10:3 and 1:3:3 and NR1a/NR2A) had significantly different affinities for Ca\textsuperscript{2+}. The NR1a/NR2A/NR2C 1:3:3 receptor population had the highest Ca\textsuperscript{2+} affinity, $K_i = 16.4 \pm 7.6$ mM. The NR1a/NR2A receptor had a $K_i$ for Ca\textsuperscript{2+} of $121 \pm 24$ mM, while the NR1a/NR2A/NR2C 1:10:3 receptor population had a Ca\textsuperscript{2+} affinity, intermediate between these values, of $K_i = 63 \pm 21$ mM.

A comparison between the pharmacological specificity for the NR1a/NR2A/NR2C 1:10:3 and 1:3:3 receptor populations, for the six ligands examined, yielded a correlation coefficient of $r = 0.993$, $p < 0.0001$ (Figure 2.3.20A), with only the values for ketamine and Ca\textsuperscript{2+} significantly different. The converse occurred, with a comparison of the NR1a/NR2A/NR2C 1:10:3 receptor population and the recombinant NR1a/NR2A receptor only $[^3]$H]MK801 and ketamine provided values that were not significantly different. A rank order of correlation for the pharmacological specificity of the six compounds tested, for these receptors produced a correlation coefficient of $r = 0.9843$, $p = 0.0004$ (Figure 2.3.19B). As a comparison, a rank order of correlation between the NR1a/NR2A/NR2C 1:3:3 receptor population and NR1a/NR2A recombinant receptor, where all the ligand affinities were significantly different, yielded a correlation coefficient of $r = 0.9784$, $p = 0.0007$ (Figure 2.3.19C).
2.3.5.3 Comparison of the Pharmacological Properties between Native Cerebellar and Recombinant NMDA Receptors

The pharmacological specificity of the recombinant NR1a/NR2A/NR2C receptors was compared to that found for the native cerebellar NMDA receptor and the correlation co-efficients determined.

Native cerebellar vs. recombinant NR1a/NR2A/NR2C 1:3:3 receptor

From Table 2.3.4 it can be seen that the dissociation constants for \(^3\)HMK801 binding to native cerebellar receptors and the cloned receptor NR1a/NR2A/NR2C for the DNA transfection ratio 1:3:3 are in good agreement. For the six compounds examined, only the Zn\(^{2+}\) inhibition coefficient differed significantly (P < 0.001). A comparison of the rank order of potency for the six compounds between the cerebellar receptor and the recombinant NR1a/NR2A/NR2C 1:3:3 receptor is shown in Figure 2.3.19D. A correlation coefficient of r = 0.992 (p < 0.0001) was found.

Native cerebellar vs. recombinant NR1a/NR2A/NR2C 1:10:3 receptor

The recombinant receptor population NR1a/NR2A/NR2C 1:10:3 also had a K\(_p\) value which was not significantly different from the native cerebellar receptor. However, further examination of the pharmacology of the NR1a/NR2A/NR2C 1:10:3 receptor, by displacement with a range of compounds, yielded significant differences for the inhibition constants of ketamine and Ca\(^{2+}\), as well as Zn\(^{2+}\) when these constants were compared to those for the native cerebellar receptor (Tables 2.3.6 and 2.3.1 respectively). The native cerebellar NMDA receptor had a 2.5-fold lower affinity for ketamine, and conversely a 10-fold higher affinity for Ca\(^{2+}\) than the recombinant NR1a/NR2A/NR2C 1:10:3 receptor population. When a rank order of potency for all six compounds was plotted for these receptors, as shown in Figure 2.3.19E, the curve gave a correlation coefficient of r = 0.9807 (p = 0.0006). This was a lower value than that found for the correlation curve comparing the cerebellar receptor and the cloned receptor NR1a/NR2A/NR2C 1:3:3.
TABLE 2.3.1 Comparison of the pharmacological specificity of \[^{3}H\]MK801 binding to native NMDA receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mouse Cerebellum</th>
<th>Mouse Forebrain</th>
</tr>
</thead>
<tbody>
<tr>
<td>[^{3}H]MK801</td>
<td>22.3 ± 9.0 nM(^a)</td>
<td>5.4 ± 0.2 nM(^a)</td>
</tr>
<tr>
<td>Tenocyclidine</td>
<td>1603 ± 145 nM(^b)</td>
<td>75 ± 30 nM(^b)</td>
</tr>
<tr>
<td>Ketamine</td>
<td>6868 ± 1324 nM(^b)</td>
<td>1700 ± 200 nM(^b)</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>4.38 ± 0.9 mM(^c)</td>
<td>0.9 ± 0.1 mM(^c)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>5.89 ± 1.68 mM(^c)</td>
<td>26 ± 8 mM(^c)</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>0.26 ± 0.09 mM(^c)</td>
<td>0.33 ± 0.05 mM(^c)</td>
</tr>
</tbody>
</table>

Binding results were analysed by Inplot software. All values are the mean ± S.D. for at least n = 3 determinations as described in 2.2.22. The mouse forebrain values are from Dr. P.L. Chazot.

\(^a\) \(K_D\) for \[^{3}H\]MK801 binding.

\(^b\) \(K_I\) for the displacement of \[^{3}H\]MK801 binding.

\(^c\) IC\(_{50}\) for the displacement of \[^{3}H\]MK801 binding.
Figure 2.3.1 A typical saturation curve (A) and Scatchard transformation (B) for $[^3H]MK801$ radioligand binding to native adult cerebellar NMDA receptors

The native cerebellar receptors were assayed for $[^3H]MK801$ radioligand binding using a range of $[^3H]MK801$ concentrations and 10 µM (+)MK801 to determine non-specific binding as described in 2.2.22. Each point is determined from triplicate values for total and non-specific binding. Curves were analysed by Inplot software.
CHAPTER 2

Figure 2.3.2  Typical curves for the displacement of \[^{3}\text{H}]\text{MK801} \text{ radioligand binding from native adult cerebellar NMDA receptors by a range of unlabelled ligands}

The native cerebellar receptors were assayed using 20 nM \[^{3}\text{H}]\text{MK801} \text{ and a range of ligand concentrations as described in 2.2.22. Each point is determined from triplicate values for total and non-specific binding. Curves were analysed by Inplot software.}

Graph A:  ● —— Ketamine; ○ —— TCP

Graph B:  ● —— Mg\(^{2+}\); ○ —— Ca\(^{2+}\); ▲ —— Zn\(^{2+}\)

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Figure 2.3.3  Restriction map of the vector pBKSII+NR2C

The vector pBKSII+NR2C was the source of the NR2C cDNA for the subcloning procedure to create pCISNMDAR2C (2.3.2). The NR2C had been cloned into the EcoRI restriction site of the pBKSII+ plasmid (Kutsuwada et al., 1992).
The plasmid pBluescript KS II (+) (Stratagene Ltd. Cambridge U.K.) has a large multiple cloning site. The *E. coli* origin of replication (Col El ori) allows growth of the plasmid in any strain of *E. coli*. The ampicillin resistance gene allows selection of colonies containing the plasmid. The fl phage origin of replication (fl ori) allows ssDNA rescue of the (+) strand.
The plasmid pCIS is a mammalian expression vector containing the CMV promoter/enhancer sequences (2.1.1). The multiple cloning site has 7 unique restriction sites for subcloning. The plasmid is readily grown in *E. coli* due to the Col El origin of replication (ori) and selected for by the ampicillin resistance gene. The phage M13 origin of replication (M13 ori) allows ssDNA recovery of the (+) strand.
Figure 2.3.6  
Restriction map of the vector pCISNR2C

This construct was generated upon the subcloning of NR2C subunit cDNA into the mammalian expression vector pCIS (2.3.2).
Figure 2.3.7  Restriction enzyme analysis of pCISNR2C

Lanes:
1  Molecular weight standards
2  pBKSII+NR2C
3  pCIS
4  pCIS digested with EcoRI
5  pCISNR2C
6  pCISNR2C digested with EcoRI
7  pCISNR2C digested with SalI
8  pCISNR2C digested with Scal
HEK 293 cells were transfected with pSV-β-galactosidase and cultured for 45 h. They were then stained with X-gal as described in 2.2.18. Photographs were taken on an inverted microscope, the film was developed as normal. The blue patches demonstrate transfected cells.
Figure 2.3.9 Immunoblot demonstrating the expression of recombinant NR2C subunit

Cell homogenates were prepared from HEK 293 cells transfected with pCISNR2C. The position of the molecular weight markers (kDa) are shown on the left.

Lanes:
1-2 Probed with anti-NR2C (1208-1218)
3-4 Probed with anti-Cys-NR2C (1-14)
5-6 Probed with anti-NR2C (1208-1218)

2 and 4 The antibody was pre-incubated overnight at 4°C with the respective peptide that was used for antibody production.

5 Cell homogenates incubated at 37°C for 4 h prior to analysis.
6 Cell homogenates treated with N-glycanase for 4 h at 37°C.
Figure 2.3.10  Restriction map of the vector pCISNR1a

The construct used in HEK 293 cell transfections for transient expression of the rat NR1a subunit (Chazot et al., 1992).
### Table 2.3.2 The effect of DNA ratios on $[^3]$HMK801 binding to HEK 293 cells co-transfected with pCISNR1a and pCISNR2C

HEK 293 cells were co-transfected with NR1a and NR2C clones by the Ca$^{2+}$-phosphate method and cultured for 40 h. The cells were harvested and assayed in triplicate for $[^3]$HMK801 binding activity using a single concentration of 50 nM. Each determination for the individual ratios was carried out on a separate HEK 293 cell transfection.

* Results are expressed with respect to $[^3]$HMK801 binding to NR1a alone which was always carried out in parallel.
FIGURE 2.3.11 Immunoblot demonstrating the expression of NR1a and NR2C subunits following the co-transfection of the respective clones in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1a and pCISNR2C with a DNA ratio of 1:10. Immunoblotting was carried out as described in 2.2.23. Molecular weight markers are shown on the left.

Lanes:
1-2 Probed with anti-NR1a (911-920)
3-4 Probed with anti-NR2C (1208-1218)
2-3 The antibody was pre-incubated overnight at 4°C with the respective peptide used for antibody production
### Table 2.3.3 Typical total and non-specific $[^3]$H]MK801 radioligand binding values to the recombinant NR1a/NR2C 1:10 receptor

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>15</td>
<td>186</td>
<td>152</td>
<td>1.22</td>
</tr>
<tr>
<td>30</td>
<td>268</td>
<td>213</td>
<td>1.26</td>
</tr>
<tr>
<td>40</td>
<td>732</td>
<td>592</td>
<td>1.23</td>
</tr>
<tr>
<td>50</td>
<td>861</td>
<td>725</td>
<td>1.18</td>
</tr>
</tbody>
</table>

HEK 293 cells were co-transfected with NR1a and NR2C subunit cDNAs and cultured for 40 h. Following harvesting and membrane preparation, the recombinant receptors were assayed using a range of $[^3]$H]MK801 concentrations and 10 μM (+)MK801 to determine non-specific binding as described in 2.2.22. Each total and non-specific binding point was determined from triplicate values. Each set of values was taken from a separate assay on separate transfection.
Figure 2.3.12  A typical curve for the displacement of $[^3]$MK801 radioligand binding by unlabelled (+)MK801 from recombinant NR1a/NR2C 1:10 receptors

HEK 293 cells co-transfected with NR1a and NR2C subunit DNA were cultured for 40 h. Following harvesting and membrane preparation, the recombinant receptors were assayed by displacement, using a range of (+)MK801 concentrations and 50 nM $[^3]$HMK801 as described in 2.2.22.
Figure 2.3.13  Restriction map of the vector pCISNR2A

The construct used in HEK 293 cell transfections for transient expression of the mouse NR2A subunit (Cik et al., 1993).
Receptor | $K_D$ (nM) | $B_{\text{max}}$ (fmol/mg protein)
--- | --- | ---
Mouse Forebrain$^a$ | 5.4 ± 0.2 | 1809 ± 157
Mouse Cerebellum | 22.0 ± 9.0 | 552 ± 215
NR1a$^a$ | 8.8 ± 2.5 | 113 ± 37
NR1a/NR2A$^b$ 1:3 + AP5 | 7.0 ± 2.4 | 976 ± 289
NR1a/NR2C 1:10 | 346 ± 158$^c$ | 1065 ± 405
NR1a/NR2A/NR2C 1:10:3 + AP5 | 13.0 ± 6.0 | 962 ± 77
NR1a/NR2A/NR2C 1:3:3 + AP5 | 22.0 ± 5.0 | 486 ± 190
NR1a/NR2A/NR2C 1:3:10 + AP5 | NS$^d$ | ND$^d$

Table 2.3.4 The dissociation constants for the binding of [H]MK801 to native and recombinant NMDA receptors

Binding results were analyzed by Inplot software. All values are means ± S.D. for at least $n = 3$ determinations.

$^a$ Results from Chazot et al. (1992). $^b$ Results from Cik et al. (1993).

$^c$ The $K_D$ was determined by displacement with unlabelled MK801.

$^d$ NS is non-saturating and ND is not determined.
HEK 293 cells were co-transfected with NR1a, NR2A and NR2C subunit cDNAs with a DNA ratio of 1:10:3 and cultured for 40 h. Following harvesting and membrane preparation the recombinant receptors were assayed using a range of $[^3$H]MK801 concentrations and 10 μM (+)MK801 to determine non-specific binding as described in 2.2.22. Each point is determined from triplicate values for total and non-specific binding. Curves were analysed by Inplot software.
CHAPTER 2

**Graph A**

**Graph B**


-bound $[^3]H$MK801 Concentration (nM)

-bound/Free

-bound

HMKK 801 Concentration (nM)
HEK 293 cells were co-transfected with NR1a, NR2A and NR2C subunit DNA with a DNA ratio of 1:3:3 and cultured for 40 h. Following harvesting and membrane preparation the recombinant receptors were assayed using a range of [H]MK801 concentrations and 10 μM (+)MK801 to determine non-specific binding as described in 2.2.22. Each point is determined from triplicate values for total and non-specific binding. Curves were analysed by Inplot software.
Figure 2.3.16 Immunoblot demonstrating the expression of NR1a, NR2A and NR2C following the co-transfection of the respective clones in HEK 293 cells

Cell homogenates were prepared from HEK 293 cells co-transfected with pCISNR1a, pCISNR2A and pCISNR2C, with the DNA ratios of 1:10:3, 1:3:3 and 1:3:10. Immunoblotting was carried out as described in 2.2.23.

Lanes:
1-3 Probed with anti-NR1a (911-920)
4 Molecular weight markers; 205 kDa, 97 kDa and 68 kDa
5-7 Probed with anti-NR2A (1432-1442)
8-10 Probed with anti-NR2C (1208-1218)

1, 5, 8 HEK 293 cells transfected with a DNA ratio of 1:10:3
2, 6, 9 HEK 293 cells transfected with a DNA ratio of 1:3:3
3, 7, 10 HEK 293 cells transfected with a DNA ratio of 1:3:10
Table 2.3.5 The percentage cell death following transient expression of NMDA receptor subtypes in HEK 293 cells

HEK 293 cells were transfected as described in 2.2.17 and incubated for 24 h in the absence of APV. Cell death was assayed by a lactate dehydrogenase (LDH) release assay as described in 3.2.11. The values were normalised to take account of the efficiency of transfection. Triplicate values were determined per transfection and the results are the mean ± S.D. for n = 3 separate transfections. The results are courtesy of Dr. M. Cik.
Table 2.3.6  
Comparison of the pharmacological specificity of 
[^3H]MK801 binding to recombinant NMDA receptors

Binding results were analysed by Inplot software. The transfected cells were incubated in the presence of AP5 (400 μM) for all the DNA ratios (1:3, 1:10:3, 1:3:3) used. The values were determined as described in 2.2.22. All values are mean ± S.D. for at least n = 3 determinations from at least two separate transfections. The NR1a/NR2A (1:3) results are from Dr. P.L. Chazot.

A) Recombinant receptor NR1a/NR2A 1:3
B) Recombinant receptor NR1a/NR2A/NR2C 1:10:3
C) Recombinant receptor NR1a/NR2A/NR2C 1:3:3
A) NR1a/NR2A 1:3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory Constant</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenocyclidine</td>
<td>64 ± 32 nM</td>
<td>1.67 ± 0.79</td>
</tr>
<tr>
<td>Ketamine</td>
<td>2600 ± 500 nM</td>
<td>1.08 ± 0.15</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>19 ± 7 mM</td>
<td>0.83 ± 0.09</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>121 ± 24 mM</td>
<td>0.79 ± 0.10</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.53 ± 0.01 mM</td>
<td>1.57 ± 0.49</td>
</tr>
</tbody>
</table>

B) NR1a/NR2A/NR2C 1:10:3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory Constant</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenocyclidine</td>
<td>1716 ± 233 nM</td>
<td>0.98 ± 0.06</td>
</tr>
<tr>
<td>Ketamine</td>
<td>2740 ± 1102 nM</td>
<td>1.04 ± 0.26</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>5.7 ± 1.9 mM</td>
<td>1.03 ± 0.33</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>63.3 ± 21.4 mM</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.89 ± 0.22 mM</td>
<td>1.02 ± 0.26</td>
</tr>
</tbody>
</table>
### C) NR1a/NR2A/NR2C 1:3:3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory Constant</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenocyclidine</td>
<td>$1609 \pm 306$ nM</td>
<td>$1.23 \pm 0.37$</td>
</tr>
<tr>
<td>Ketamine</td>
<td>$7102 \pm 925$ nM</td>
<td>$0.68 \pm 0.08$</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>$6.32 \pm 3.3$ mM</td>
<td>$0.77 \pm 0.05$</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>$16.4 \pm 7.6$ mM</td>
<td>$0.85 \pm 0.22$</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>$1.10 \pm 0.1$ mM</td>
<td>$1.47 \pm 0.64$</td>
</tr>
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</table>
Figure 2.3.17  Typical curves for the displacement of $[^3]$MK801 radioligand binding from recombinant NR1a/NR2A/NR2C 1:10:3 receptors by a range of unlabelled ligands

The recombinant NR1a/NR2A/NR2C 1:10:3 receptors were assayed using 20 nM $[^3]$MK801 and a range of ligand concentrations as described in 2.2.22. Each point is determined from triplicate values for total and non-specific binding. Curves were analysed by Inplot software.

Graph A:  • ——— Ketamine; ○ ——— TCP
Graph B:  • ——— Mg$^{2+}$; ○ ——— Ca$^{2+}$; ▲ ——— Zn$^{2+}$
Total binding was 3500-4000 dpm and non-specific binding was 900-1400 dpm.

The range between 100% and 0% binding was approximately 2500 dpm.
Figure 2.3.18  Typical curves for the displacement of $[^3H]MK801$
radio ligand binding from recombinant
NR1a/NR2A/NR2C 1:3:3 receptors by a range of
unlabelled ligands

The recombinant NR1a/NR2A/NR2C 1:3:3 receptors were assayed using 20 nM
$[^3H]MK801$ and a range of ligand concentrations as described in 2.2.22. Each
point is determined from triplicate values for total and non-specific binding.
Curves were analysed by Inplot software.
Graph A:  ● —— Ketamine; ○ —— TCP
Graph B:  ● —— Mg$^{2+}$; ○ —— Ca$^{2+}$; ▲ —— Zn$^{2+}$
Total binding was 3500-4000 dpm and non-specific binding was 900-1400 dpm. The range between 100% and 0% binding was approximately 2500 dpm.
Figure 2.3.19  Correlation of the rank order of potency of pharmacological specificity between recombinant and native adult cerebellar NMDA receptors

A-C show the correlation of affinity constants between recombinant receptor subtypes. D and E demonstrate the correlation of affinity constants between native cerebellar NMDA receptors and triple subunit recombinant NMDA receptor populations. The dotted lines indicate the 95% confidence limits for each correlation.

1, MK801; 2, TCP; 3, Ketamine; 4, Mg²⁺; 5, Ca²⁺; 6, Zn²⁺.
CHAPTER 2

-Log_{10}[Inhibitory constant] (M) for NR1a/NR2A/NR2C 1:10:3

-Log_{10}[Inhibitory constant] (M) for NR1a/NR2A 1:10:3
CHAPTER 2

![Graphs showing log-log plots of inhibitory constant against cerebellum for NR1a/NR2A/NR2C ratios 1:3:3 and 1:10:3.](image)
The aim of this chapter was to establish a recombinant model of the cerebellar NMDA receptor population. This was characterized using radioligand binding and immunochemistry and a comparison was made with the native cerebellar NMDA receptor. Previous work has demonstrated the successful transient co-expression of rat NR1a and mouse NR2A subunits in HEK 293 cells using the pCIS mammalian expression vector (Chazot et al., 1992; Cik et al., 1993). The studies detailed below also employed the mouse NR2C cDNA, as in situ hybridization studies showed the restricted expression of NR2C mRNA in cerebellum (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993).

### 2.4.1 Characterization of the native cerebellar NMDA receptor

Initial studies characterized the native mouse cerebellar NMDA receptor. Radioligand binding using $[^3]H$MK801 gave a significantly different $K_D$ for mouse cerebellum compared to mouse forebrain (Table 2.3.1). Further pharmacological characterization of the mouse cerebellar NMDA receptor highlighted the differences between forebrain and cerebellar NMDA receptors (Table 2.3.1). This concurred with the report of Ebert et al. (1991) which showed that the pharmacology of the cerebellar $[^3]H$MK801 binding sites was significantly different from $[^3]H$MK801 binding in the cortex. Electrophysiological studies have also demonstrated differences between forebrain and cerebellar NMDA receptors, for example, Priestly and Kemp (1993) showed significant differences in the kinetics of L-glutamate and glycine binding between rat cortical neurones and rat cerebellar granule cells.

### 2.4.2 Expression of the single NR2C subunit

Prior to carrying out the transfection studies using the NR2C clone it was necessary to subclone the NR2C cDNA into the mammalian expression vector.
pCIS. Following the subcloning, restriction enzyme analysis was carried out to determine the correct orientation of the cDNA. The subcloning strategy was determined by a number of factors; the restriction sites in the multicloning site of the pCIS vector and the restriction sites in the cloning region of the pBKSII+NR2C construct and the unsequenced state of the 5' and 3' untranslated regions of the NR2C cDNA. The non-directional cloning approach using the single restriction enzyme *EcoRI*, was unaffected by the presence or absence of an *EcoRI* site in the untranslated, unsequenced 5' and 3' regions (2.3.2). When the NR2C clone had been successfully subcloned into the pCIS vector transfection of HEK 293 cells allowed the transient expression of the NR2C subunit protein (2.3.3).

Initial transfections with the pCISNR2C construct only were carried out to characterize the NR2C polypeptide. This was detected by subunit-specific polyclonal antibodies, to the N- and C-terminus of the protein. Immunoblotting showed a single immunoreactive band of $M_r$ 145000, while N-deglycosylation of the NR2C polypeptide generated a single band of $M_r$ 133000. The molecular mass of the N-deglycosylated NR2C polypeptide agreed well with the predicted molecular mass of 133513 Da from the cDNA sequence (Kutsuwada *et al.*, 1992). Five putative N-glycosylation sites were predicted in the N-terminal region of the NR2C subunit, and one possible N-glycosylation site was seen in the C-terminal region (Ishii *et al.*, 1993). No $[^3]H$MK801 binding activity was seen in HEK 293 cell homogenates singly transfected with pCISNR2C, this was not unexpected. The inability of NR2 subunits to form functional receptors has been widely reported for electrophysiological studies following expression in Xenopus oocytes or HEK 293 cells (e.g. Meguro *et al.*, 1992; Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992). Cik *et al.* (1993) also reported a lack of $[^3]H$MK801 binding in HEK 293 cells expressing only the NR2A subunit.
2.4.3 Co-expression of the NR1a and NR2C subunits

Following the characterization of the NR2C polypeptide, co-transfection experiments with pCISNR1a and pCISNR2C constructs were performed. These experiments with the NR1a and NR2C clones used cDNAs from rat and mouse respectively. However, the rat and mouse NR1a subunit proteins are 99.8% identical, with only two conserved substitutions in the C-terminal region, while the NR2C protein sequences are more diverse, having 94.8% identity between rat and mouse. Therefore, the heteromeric receptor NR1a/NR2C may be considered a mouse NMDA receptor. The presence of both subunits in co-transfected HEK 293 cell cultures was confirmed by immunoblotting. Moreover, radioligand binding using $[^3H]$MK801 following co-transfection with pCISNR1a and pCISNR2C was detected, although at low levels. The co-assembly of the NR1 and NR2C subunits was shown by immunoprecipitation (Chazot et al., 1994). The functionality of these receptors was shown by L-glutamate activated Ca$^{2+}$-influx (Cik et al., 1995).

Due to the low level of $[^3H]MK801$ binding and from prior studies on the NR1a/NR2A recombinant receptor (Cik et al., 1993), the optimal DNA ratio of NR1a:NR2C for transfection was established. This was determined by the maximum level of $[^3H]MK801$ binding (2.3.4.1). The optimum DNA ratio was found to be 1:10 for NR1a:NR2C. That the optimal DNA ratio was not 1:1 was possibly due to differences in efficiency of transcription and translation for the different subunits, the subunit stoichiometry of the NMDA receptor or both. However, the maximal level of $[^3H]MK801$ binding for the NR1a/NR2C receptors transfected with a 1:10 cDNA ratio was only 2-fold greater than the radioligand binding seen in homomeric NR1a receptors, see Table 2.3.2. This compared unfavourably with the 10-fold increase in $[^3H]MK801$ binding activity for optimal co-expression of NR1a/NR2A (Cik et al. 1993). Attempts to determine the $K_D$ of $[^3H]MK801$ binding to NR1a/NR2C cloned receptors by saturation curves were unsuccessful as the specific binding was not saturable at
concentrations of $[^\text{H}]$MK801 up to 70 nM. The MK801 affinity of the NR1a/NR2C receptor was determined by displacement and was found to be over 10-fold lower than the value found for native cerebellar NMDA receptors (Table 2.3.4). This compares well with the studies of Laurie and Seeburg (1994a).

2.4.4 Co-expression of NR1a and NR2A and NR2C subunits

Due to the low affinity for $[^\text{H}]$MK801 of the recombinant NR1a/NR2C receptor, triple transfections with NR1a, NR2C and NR2A clones were carried out. As previously, the effects of different DNA ratios were investigated. The DNA ratios used for the triple transfections were 1:10:3, 1:3:3 and 1:3:10 of NR1a:NR2A:NR2C (2.3.5). The expression of all three polypeptides was verified by immunoblotting. The $[^\text{H}]$MK801 radioligand binding to the recombinant receptor populations was initially assayed by full saturation curves as opposed to single point binding since the presence of the NR1a, NR2A and NR2C subunits may give formation of heterogenous populations of recombinant receptors, i.e. NR1a/NR2A and NR1a/NR2C and NR1a/NR2A/NR2C, or any combination thereof. Single point $[^\text{H}]$MK801 binding would have been unable to distinguish if there was a predominant single population of cloned receptors, or whether all the receptor combinations were equally available. Saturation binding analysis on HEK 293 cell homogenates transfected with the DNA ratios of 1:10:3 and 1:3:3 of NR1a:NR2A:NR2C DNA yielded receptor populations with an apparently single high affinity $[^\text{H}]$MK801 binding site. The $K_D$ values differed significantly from the determined MK801 affinity for the NR1a/NR2C recombinant receptor but correlated with the $K_D$ value for native cerebellar NMDA receptors (Table 2.3.4). However, while the dissociation constant for the NR1a/NR2A/NR2C 1:3:3 receptor population differed significantly from that of the NR1a/NR2A recombinant receptor, the $K_D$ for the 1:10:3 receptor population did not. The receptor population generated by transfection with a DNA ratio of 1:3:10 of NR1a:NR2A:NR2C when analysed by MK801 binding showed a
greater similarity to the cloned NR1a/NR2C receptor population. The 1:3:10 receptor population was non-saturating at high concentrations of $[^3H]MK801$ with a low signal to noise ratio. Attempts to determine the MK801 affinity by displacement, as for the NR1a/NR2C receptor, were unsuccessful. This could have been due to minor subpopulations of NR1a/NR2C and NR1a/NR2A receptors. These minor subpopulations could be sufficient to effect the already low levels of $[^3H]MK801$ binding to the 1:10:3 NR1a/NR2A/NR2C receptor population so that meaningful binding data could not be determined.

Further pharmacological investigation was carried out on the cloned receptor populations generated by the cDNA ratios of 1:10:3 and 1:3:3 (2.3.5.2) in order to ascertain the closest resemblance to the native cerebellar receptors. The same ligands as used for investigation of the cerebellar NMDA receptor were employed. Both cloned receptor populations were best fit by a single predominant receptor type, no displacement curve could be significantly better fitted to a multiple binding site model. However, a minor population of receptors may not have been distinguished by the radioligand and displacement studies. The NR1a/NR2A/NR2C receptor populations of DNA ratio 1:10:3 and 1:3:3 were very similar with a correlation co-efficient of 0.993. A comparison of these receptor populations with the recombinant NR1a/NR2A receptor yielded correlation co-efficients of $r = 0.9843$ and $r = 0.9784$ for NR1a/NR2A/NR2C 1:10:3 and 1:3:3 respectively. The rank order of affinity of the six ligands for the recombinant NMDA receptor populations was the same. Of the six ligands used only two, ketamine and Ca$^{2+}$, gave significantly different $K_i$ values between the 1:3:3 and 1:10:3 NR1a/NR2A/NR2C receptor populations. The reduced Ca$^{2+}$ affinity of the 1:10:3 receptor population (as compared to the 1:3:3 NR1a/NR2A/NR2C receptor population) presumably has implications for its greater cytotoxic effect (see below). However, the most interesting observation involves the PCP binding site, where the non-competitive antagonists, MK801, TCP and ketamine interact with the NMDA receptor. The inclusion of NR2C
subunits in the recombinant receptors significantly altered the TCP displacement constant compared to the value seen for the NR1a/NR2A receptor. Both NR1a/NR2A/NR2C receptor populations had an affinity for TCP over 25-fold lower than the NR1a/NR2A receptor. However, the 1:10:3 receptor population had an affinity for ketamine which was similar to the NR1a/NR2A receptor, whilst the 1:3:3 receptor population had a 2.5-fold lower affinity for ketamine, see Table 2.3.5.

2.4.4.1 Comparison of the recombinant NR1a/NR2A/NR2C receptors and the native cerebellar NMDA receptor

A comparison of the \[^{3}H\]MK801 affinity of both 1:10:3 and 1:3:3 recombinant NR1a/NR2A/NR2C receptor populations with the native cerebellar NMDA receptor showed that both recombinant receptors had $K_d$ values which were not significantly different from that of the native cerebellar receptor. Further comparison of the inhibition coefficients of the six compounds examined showed that a rank order of affinity for the 1:3:3 NR1a/NR2A/NR2C cloned receptor and the native cerebellar NMDA receptor yielded a correlation coefficient, $r = 0.992$. Only the inhibition coefficient values for Zn$^{2+}$ differed significantly between the native cerebellar receptor and the 1:3:3 NR1a/NR2A/NR2C cloned receptor. However, comparison of the second recombinant receptor population NR1a/NR2A/NR2C 1:10:3 to native cerebellar NMDA receptors indicated significant differences in the affinities of ketamine, Ca$^{2+}$ and Zn$^{2+}$. Native cerebellar NMDA receptors had a 10-fold lower IC$_{50}$ value for displacement of \[^{3}H\]MK801 binding by Ca$^{2+}$, whereas the NR1a/NR2A/NR2C 1:10:3 recombinant receptor had a 2.5-fold higher affinity for ketamine and over 3-fold higher affinity for Zn$^{2+}$. A rank order of correlation between the recombinant NR1a/NR2A/NR2C 1:10:3 receptor population and the native cerebellar NMDA receptor gave a correlation co-efficient of $r = 0.9807$. This value was lower than that found for the native cerebellar NMDA receptor.
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and the 1:3:3 NR1a/NR2A/NR2C receptor population. Thus indicating the 1:3:3 NR1a/NR2A/NR2C receptor population is a better model of the native cerebellar NMDA receptor population.

The variation in the Zn\(^{2+}\) affinity of both NR1a/NR2A/NR2C cloned receptor populations and the native cerebellar NMDA receptor was interesting. All the recombinant receptor studies described used the NR1a splice variant of the NR1 subunit. This splice variant was the most abundant as determined in the original cloning studies (Sugihara et al., 1992). However, further in situ hybridisation studies found a regional variation of the NR1 splice variants. The NR1 isoforms lacking the N-terminal insert (NR1a, NR1c, NR1d, NR1e) were most prominently seen in the forebrain and rostral structures, whereas the splice variants containing the N-terminal insert (NR1b, NR1f, NR1g, NR1h) were more prominent in the cerebellum and brainstem. More particularly, the NR1g isoform appeared to be the most pronounced in the cerebellum (Laurie and Seeburg, 1994b). Moreover, studies on homomeric NR1 receptors demonstrated the N-terminal insert modulated NMDA receptor interaction with Zn\(^{2+}\) (Hollmann et al., 1993). The inhibition of the homomeric NR1 receptors was determined as a percentage of the control electrophysiological response to glutamate and glycine. The homomeric NR1a and NR1g receptor responses were inhibited by 10.5% and 20.5% respectively by 100 μM of Zn\(^{2+}\) (Hollmann et al., 1993). This concurs with the lower Zn\(^{2+}\) affinity seen in the recombinant NR1a/NR2A/NR2C receptors compared to the native cerebellar NMDA receptor. However, co-expression of NR1g isoform with NR2A and NR2C will clarify this.

Immunoprecipitation assays and native PAGE substantiate the pharmacological finding suggesting the co-association of all three subunit types (Chazot et al., 1994). Immunoprecipitation of the NR1a subunit, following triple transfections of NR1a, NR2A and NR2C, allowed the detection of both NR2A and NR2C on immunoblots. Moreover, immunoprecipitation with antibodies against NR2C also precipitated the NR2A subunit, demonstrating the co-
association of NR2A and NR2C subunits (Chazot et al., 1994). Thus the co-
assembly of NR1a/NR2A/NR2C following transfection at a defined DNA ratio
forms a receptor population with properties similar to the native cerebellar
NMDA receptor which suggests the co-existence of three subunit types in
cerebellar NMDA receptors. This co-association is consistent with both in situ
hybridization studies which show the co-localization of NR1, NR2A and NR2C
mRNA in adult cerebellar granule cells (Monyer et al., 1994) and the
observations of Wafford et al. (1993) who demonstrated the preferential co-
assembly of NR1/NR2A/NR2C subunits expressed in Xenopus oocytes. The
ability of three subunit types to co-exist in a native NMDA receptor complex
was suggested by Sheng et al. (1994) by immunoprecipitation studies with
subunit-selective antibodies. These studies inferred the co-association of NR1,
NR2A and NR2B subunits in rat cortex NMDA receptors.

2.4.5 Neurotoxic effects of the recombinant receptor populations

Interestingly, the co-transfection of NR1a and NR2C clones did not result
in cell death, unlike co-transfection of the NR1a and NR2A clones (Cik et al.,
1993), although both heteromeric receptors had a similar range of $B_{\text{MAX}}$
values. This could result from a decreased sensitivity to L-glutamate or reduced Ca$^{2+}$
permeability. Electrophysiological studies showed that the NR1a/NR2C receptors
had a greater sensitivity to L-glutamate than the NR1a/NR2A receptors (e.g.
Kutsuwada et al., 1992). Recent studies have suggested that Ca$^{2+}$ may act as a
permeant blocker of both NR1/NR2A and NR1/NR2C channels with the
NR1/NR2C channel having the stronger block (Burnashev et al., 1995). The
voltage-dependent Mg$^{2+}$ block of NMDA receptors was thought unlikely to play
a role, although the cell culture medium contained Mg$^{2+}$ (0.7 mM) in
physiologically relevant concentrations. However, the resting membrane
potentials of HEK 293 cells are in the range of -15 to -50 mV (Cik et al., 1993).
At these potentials the voltage-dependent Mg$^{2+}$ block is strongly reduced
An interesting observation was that altering the DNA ratio in the triple transfection studies influenced the properties of the expressed receptor. If the amount of NR2A clone was increased, a receptor with properties similar but not identical to the NR1a/NR2A receptor resulted. Increasing the NR2C clone resulted in a receptor with a low affinity for \[^{3}H\]MK801, similar to the NR1a/NR2C receptor. None of the recombinant receptor populations studied were better fit by 2-site displacement curves compared to a 1-site model, although a minor subpopulation of NMDA receptors is unlikely to be detected by the techniques used. The triply transfected cells, for all the DNA ratios, resulted in some cell death, which was quantified (2.3.5). The cytotoxic properties of the transiently expressed NMDA receptor populations were influenced by the ratio of the clones used for the transfection (Table 2.3.5). Increasing the quantity of NR2A cDNA used for transfection or decreasing the amount of NR2C cDNA increased the percentage cell death. These results may be explained in two ways. Increasing the amount of NR2C DNA used for transfection from 1:3:3 to 1:3:10 may have forced an increased number of NR2C polypeptides into the assembled receptor. The NR2C subunits in the NR1a/NR2A/NR2C channel may then modulate the Ca\(^{2+}\) influx, in a manner similar to that proposed for the NR1a/NR2C receptor (see above). The converse could also be possible. An increased amount of NR2A cDNA used for transfection could force an increased number of NR2A polypeptides in the assembled receptor. This theory implies that the stoichiometry of the assembled receptors is not predetermined, but can be influenced by the relative amounts of each subunit available. An alternative (and perhaps more likely) explanation is that the stoichiometry of the NMDA receptor is predetermined. Therefore the relative amounts of each subunit do not have any influence on assembly of the NR1a/NR2A/NR2C receptor and the alteration in cytotoxicity of the expressed receptor populations is due to a changing subpopulation of NR1a/NR2A
receptors. The NR1a/NR2A/NR2C stoichiometry may be determined by subunit-subunit interactions as for the nAChR (reviewed in Green and Millar, 1995). Furthermore, an excess amount of an NR2 subunit could then co-assemble with NR1a to form a minor population of receptors containing only two polypeptide types. These receptors could have a disproportionate influence on the cytotoxicity of the cloned receptor population. It had been previously noted that the NR1a/NR2A receptor was highly cytotoxic to the HEK 293 cells, while the NR1a/NR2C receptor had no significant effect on cell viability. The finding that NR1 subunits will preferentially combine with available NR2 subunits to form heteromeric receptors (e.g. Ishii et al., 1993), and the report of Behe et al. (1995) that recombinant NMDA receptors expressed in *Xenopus* oocytes contain two copies of the NR1 subunit suggest that the assembly of NMDA subunits is not a random process. This is supported by the report of Wafford et al. (1993) which suggested the preferential co-assembly of three NMDA receptor subunits in *Xenopus* oocytes. The controlled stoichiometry of the *Torpedo* nAChR would also suggest that the assembly of ligand-gated ion channel subunits is not a random process (Green and Millar, 1995).

In summary, the NR2C clone was successfully subcloned into the mammalian expression vector pCIS and transiently expressed in HEK 293 cells. Optimal conditions for the transient expression of NR1a/NR2C and later, NR1a/NR2A/NR2C receptors were established. The pharmacological specificity of $[^3H]MK801$ binding to the recombinant NMDA receptors was investigated and compared to that previously established for the native cerebellar NMDA receptor. It was found that transient expression of three subunit types following transfection with a DNA ratio of NR1a:NR2A:NR2C 1:3:3 yielded a receptor population with properties that closely resembled the cerebellar NMDA receptor. Therefore, it may be concluded that the native cerebellar NMDA receptor is composed of three subunit types, NR1, NR2A and NR2C which co-exist in an unknown stoichiometry.
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Site-directed in vitro mutagenesis of the NR2C subunit
3.1 INTRODUCTION

3.1.1 Site-directed in vitro mutagenesis

Classically, mutations in DNA were produced by chemical or physical mutagenic agents. These acted upon the whole organism in a non-specific manner. There were serious disadvantages to this technique; any gene could be mutated so the frequency of the desired mutation could be very low. Long selection and screening procedures were generally needed with the uncertainty that the desired phenotype may not have been caused by a change in the gene of interest. Also, the type of mutation (insertion, deletion or base change) and where in the gene it occurred was unknown.

Work in the 1970s on the genome of the bacteriophage ΦX174 showed the feasibility of introducing specific changes in DNA (Hutchinson and Edgell, 1971). Now, with the ability to manipulate and clone single genes, the availability of high purity DNA modifying enzymes and the routine synthesis of oligonucleotides, it is possible to specifically change any given base in a cloned DNA sequence. The simplest way to create mutations is to utilize unique restriction enzyme sites within the DNA sequence of interest to create deletion mutations. There are also two-step polymerase chain reaction (PCR) methods to create point mutations (Ho et al., 1989; Mori et al., 1992). However, the commonest method is site-directed in vitro mutagenesis, using synthesised oligonucleotides to create specific changes in the DNA sequence of interest. A range of mutation types are feasible, which include, insertions, deletions, point changes of a single base or multiple bases.

The original method of oligonucleotide-directed mutagenesis used a single oligonucleotide with a single base mismatch (Hutchinson et al., 1978; Gilliam and Smith, 1979). This was annealed to a ssDNA template and used to prime DNA synthesis in vitro, itself being incorporated into the resulting heteroduplex molecule. This heteroduplex is used to transform E. coli. However, the efficiency
of mutation was very low and the resulting plaques needed to be screened by nucleic acid hybridization, with positives then being sequenced. The non-methylated, *in vitro* synthesised, mutant strand was preferentially susceptible to mismatch repair by the *E. coli* DNA repair machinery. This was overcome by using special host strains of *E. coli*, e.g. *mutL*, *mutS* and *mutH*, which prevented methyl-directed repair of base mismatches (Kramer *et al.*, 1984a). The mutant oligonucleotides susceptibility to 5'→3' exonuclease activity was reduced by using a second primer which hybridized to the 5' side of the mutant oligonucleotide (Zoller and Smith, 1984). This second oligonucleotide was phosphorylated and used to initiate DNA synthesis.

Increased efficiency of mutagenesis was achieved by suppressing the growth of non-mutant progeny. Therefore, some sort of selection for the mutant strand was required. A number of systems developed an *in vivo* selection mechanism, for example, the 'gapped duplex' method (Kramer *et al.*, 1984b). Here, the DNA of interest was cloned into an M13 vector containing an amber mutation. Therefore, the construct could only grow in an amber suppressing host. A 'gapped duplex' was formed by annealing linear wild-type M13 (no amber mutation) to the denatured construct. The mutant oligonucleotide was annealed within the gap and to the DNA of interest. The resulting heteroduplex was used to transform *E. coli* lacking an amber suppressor. Therefore, only mutant progeny should be recovered. An efficiency of up to 70% has been claimed. However, the mutational frequency was dependent upon the target sequence and the mutant oligonucleotide. Also, if further rounds of mutagenesis were necessary, the mutated DNA sequence needed to be recloned. An improved method was that of Kunkel (1985), which is currently used in the Bio-Rad Mutagen Kit. In this method the phage was grown in a specialised host prior to the mutagenesis. The host was deficient in dUTPase (dut) and uracil glycosylase (ung), allowing the replacement of thymidine bases with deoxyuridine at some positions of the DNA. Phage M13 grown in a dut ung* E. coli* host has 20-30
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uracil bases per genome, and cannot grow in a ung⁺ host, as the uracil containing strands are degraded. Therefore, a heteroduplex of parental (uracil-containing) DNA and mutant (synthesised in vitro with dTTP) DNA could yield 80% mutant progeny when plated with a wild type (ung⁺) host. This was due to the degradation of the wild-type uracil-containing strand. The efficiency of this method allows primary screening by sequencing, as opposed to nucleic acid hybridization techniques. A second round of mutagenesis is possible by growing the first mutant in a dut ung⁺ host. This method has been used to create mutants for investigation of the Ca²⁺ permeability and channel properties of the NMDA receptor (Sakurada et al., 1993). Both of the above methods relied on strand selection in vivo, which could result in loss of mutagenic efficiency and require specialized, mutant vectors or hosts. It was also possible to obtain mixed plaques containing both mutant and wild-type clones which needed further purification.

An alternative method which overcame both these problems relied on in vitro strand selection. This method depended on the observation that some restriction enzymes (e.g. Aval, BanII, HindII, NciI, PstI and PvuII) could not cleave phosphorothioated DNA (Taylor et al., 1985a). A schematic representation of the method is shown in Figure 3.1.1. The ss template DNA was primed by the mutant oligonucleotide and extended in the presence of a thionucleotide. A heteroduplex was formed, with the mutant strand phosphorothioated, by the incorporation of deoxy-cytidine-5'-[αS]-thiotriphosphate (dCTPαS). The restriction enzyme, NciI, was able to cut the template strand but not the phosphorothioated mutant strand. The parental strand was partially digested away with exonuclease III and then repolymerized, using the remaining fragments for priming (Taylor et al., 1985b; Nakamaye and Eckstein, 1986). The efficiency of mutation was 95%, allowing direct screening by sequencing. This mutagenesis technique is utilized by the Amersham Sculptor IVM system. The efficiency of mutagenesis was further improved by exonuclease digestion of excess ssDNA template. Specialized host or phage systems were not required, any plasmid
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vector which had an origin of replication for ssDNA production could be used.

This mutagenesis method has been used successfully in many studies of structure-function relationships of ligand-gated ion channels. These include an investigation of the role of the Q/R site of non-NMDA glutamate receptors in rectification and divalent ion permeability (Dingledine et al., 1992); the identification of the glycine binding site of NMDA receptors (Kuryatov et al., 1994); the structural features of NMDAR1 subunits that control current amplitude and spermidine and Zn\(^{2+}\) potentiation (Zheng et al., 1994); the permeability of neuronal \(\alpha 7\) nicotinic receptor by mutagenesis of the M2 channel domain (Bertrand et al., 1993).

This method of site-directed mutagenesis was chosen due to its proven ability for investigation of the properties of ligand-gated ion channels, the high efficiency of the mutagenesis procedure and for its lack of specialized requirements, such as particular vectors or host cells.

3.1.2 Sequencing of DNA

DNA sequencing is a fundamental capability of modern genetics. Knowledge of a DNA sequence is necessary in its own right, for example, in analysing genetic disease and the structure-function of DNA molecules. DNA sequence information allows the inference of protein sequences and it is absolutely necessary for any manipulation of DNA, such as site-directed mutagenesis or controlled restriction enzyme digest. DNA sequencing is utilized here for the verification of mutations created within the NR2C subunit.

There are two methods in current use, that of Maxam and Gilbert (1977) and the dideoxy procedure of Sanger et al. (1977). Both of these methods depend upon producing DNA fragments which differ in length. These DNA molecules are denatured and separated by vertical, high-resolution polyacrylamide gel electrophoresis. Such gels generally contain 6-20% polyacrylamide with 7-8 M urea and distinguish between DNA strands differing by a single base length.
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Urea minimises DNA secondary structure which affects electrophoretic mobility and gels are run at high temperatures (~50°C) for the same reason. The creation of the DNA fragments generally involves the inclusion of a radiolabelled nucleotide. This allows the imaging of the individual DNA fragments, separated by size, following the exposure of the gel to autoradiographic film. The sequence is read from the 'ladders' formed by the DNA bands in the adjacent, base-specific lanes. The introduction of gradient gels has improved the resolution and separation of the bands. This allows a greater amount of sequence to be read from a single gel run.

The chemical method of Maxam and Gilbert (1977), which is less used, relies on chemical reactions which specifically alter one or two bases. The altered base can then be removed from the sugar-phosphate backbone. The DNA strand is then cleaved at the sugar residue lacking a base. The method requires a DNA restriction fragment which is radiolabelled at the 3' or 5' end. The base specific reactions are limited to one residue per specific reactions are redundant, permitting confirmation of the base identifications. The five reactions allow identification of; A and G, C or T, A and C, C alone and G alone. This method sequences the actual DNA of interest, rather than a complementary copy, and is able to recognise the presence of DNA modifications, such as methylation.

The more routine method of DNA sequencing is the chain terminating, dideoxy procedure of Sanger et al. (1977). This relies on two properties of DNA polymerase; the ability to synthesise an accurate, complementary copy of a ssDNA template and to use the 2'3'-dideoxynucleoside 5'-triphosphates (ddNTPs), derivatives of the deoxyribonucleotides (dNTPs), as substrates for DNA synthesis. When the ddNTP analogue is incorporated into the synthesised strand, the 3' end lacks a hydroxyl group preventing further elongation. Extension is primed by an oligonucleotide and it is carried out in four separate reactions. Each reaction contains a low concentration of one of the ddNTPs and radiolabelled
dATP. The reactions result in DNA molecules of different lengths having a common 5' end and different 3' ends.

The original Sanger method used the Klenow fragment of DNA polymerase I., which lacks the 5'→3' exonuclease activity of the complete DNA polymerase I enzyme, and ^32P-ATP. However, the sharpness of the bands is improved by replacing the radiolabel ^32P with the weaker β-emitters ^35S or ^33P. Other improvements have been made by using different polymerases. The Klenow fragment of DNA polymerase I has a low processivity, a low rate of polymerization, 3'→5' exonuclease activity and discriminates 1000-fold against ddNTPs, as compared to the dNTPs. Alternative polymerases, such as Taq DNA polymerase, are now being used which overcome some or all of these problems. Taq DNA polymerase has a high processivity and can be used at high temperatures (65-70°C) which reduces chain termination artifacts caused by secondary structure. Other alternatives to the Klenow fragment of DNA polymerase I are Sequenase (Tabor and Richardson, 1987) and Sequenase 2.0 (Tabor and Richardson, 1989). These are modified forms of T7 DNA polymerase. The original version, Sequenase, is chemically modified so the 3'→5' exonuclease activity is lost, while the high processivity and rapid rate of synthesis of the native form is retained. Sequenase can also utilize nucleotide analogues that increase electrophoretic resolution by eliminating band compressions. Sequenase 2.0 is a deletion mutant of the native T7 DNA polymerase, again losing the 3'→5' exonuclease activity. However, the recombinant polymerase has a higher specificity and is more stable than Sequenase. Due to the high rate of processivity with Sequenase 2.0, the sequencing reactions are set up in two steps. The first step, the extension reaction, ensures efficient incorporation of the radiolabelled nucleotide, [α^35S]dATP. This is then divided into four reactions each incorporating one of the ddNTPs, the chain termination step. The elongation reaction continues until a ddNTP is incorporated.
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In this chapter the generation of point mutations in the NR2C subunit will be described and the effects characterised. The mutations of interest are indicated in Figure 3.1.2. As previously described the co-expression of NR1a and NR2C clones yielded a receptor with low affinity for the binding of $[^3H]MK801$. However, the recombinant receptor NR1a/NR2A has an affinity for $[^3H]MK801$ which is 50-fold higher. MK801 is an open channel blocker of the NMDA receptor (Wong et al., 1986; Huettner and Bean, 1988), which suggests that it binds within the pore of the NMDA receptor. The current topological model of the NMDA receptor subunits, as shown in Figure 1.4, predicts that the M2 region forms the lining of the NMDA receptor channel (Bennett and Dingledine, 1995). An alignment of the NR2A and NR2C protein sequences for the M2 region reveals only 3 amino acid differences within the predicted M2 region, with a further 2 amino acid changes just outside this area, see Figure 3.1.2. Amino acids of the NR2C subunit M2 region were mutated to the equivalent NR2A amino acids and mutant subunits were co-expressed with NR1a. Mutant and wild-type NR1a/NR2C receptors were characterised by $[^3H]MK801$ binding, immunoblotting and cytotoxicity assays.
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Figure 3.1.1  The Amersham oligonucleotide-directed \textit{in vitro} mutagenesis system

From Sculptor\textsuperscript{TM} in vitro mutagenesis system, Amersham International plc.
Figure 3.1.2 Schematic representation of the NR2C subunit showing the positions of the mutations made.
CHAPTER 3

3.2 MATERIALS AND METHODS

3.2.1 Materials

Helper phage M13KO7, M13mp18 RFI DNA and bacterial strain *E. coli. NM522* (Genotype; supE, thi, Δ(hsdMS-mcrB)5, Δ(lac-proAB), F' [proAB', lacI9, lacZΔM15]) were obtained from Pharmacia Biotech Ltd., (Hertfordshire, U.K.). T4-polynucleotide kinase, isopropyl-β-D-thiogalactopyranoside (IPTG) and deoxy-adenosine-5'-triphosphate (dATP) were purchased from Boehringer Mannheim GmbH (Lewes, Sussex, U.K.). Sequenase™ DNA sequencing kit (version 2.0) was obtained from United States Biochemical (Cleveland, Ohio, U.S.A.). Sculptor™ site-directed *in vitro* mutagenesis system, [³⁵S] deoxy-adenosine-5'-[αS]thiophosphate (dATPαS, >1000 Ci/mmol) and bacterial strain *E. coli. TG1* (Genotype; K12, Δ(lac-pro), supE, thi, hsdD5/F'traD36, proA'B', lacI9, lacZΔM15) were from Amersham International plc (Buckinghamshire, U.K.). Oligonucleotide probes were obtained from Oswell DNA Services (Edinburgh, U.K.). Thin-walled GeneAmp™ reaction tubes were purchased from Perkin-Elmer Ltd., (Buckinghamshire, U.K.). Bio-spin 30 chromatography columns were from Bio-Rad Laboratories Ltd. (Hertfordshire, U.K.). Eppendorf GELoader capillary tips were from BDH Laboratory Supplies (Leicestershire, U.K.). Urea and X-OMAT AR-5 Kodak scientific imaging film were obtained from the Sigma Chemical Co. Ltd., (Poole, Dorset, U.K.). CytoTox 96™ Non-Radioactivity Cytotoxicity Assay was obtained from Promega Corporation (Southampton, U.K.). All other materials were as described in Chapter 2 or were obtained from commercial sources.
3.2.2 Preparation of NM522 cells

A single disc of lyophilised culture was suspended in LB media (1 ml) supplemented with thiamine (1 μg/ml) and grown overnight at 37°C with vigorous shaking. An aliquot of the overnight culture was streaked on a M9 Minimal media plate supplemented with thiamine (1 μg/ml). The plates were prepared by adding 0.4% (v/v) glucose and 0.2% (v/v) 5 x M9 salts (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄, 43 mM NaCl and 9 mM NH₄Cl) to a cooled, autoclaved solution of 1.5% (w/v) agar, 0.1 mM CaCl₂ and 1 mM MgSO₄. The inoculated plate was incubated at 37°C for 18-24 h in an inverted position. An individual colony was picked and used to prepare NM522 competent cells as described in 2.2.3.

3.2.3 Preparation of plasmid ssDNA

Competent NM522 cells were transformed with pCISNMDAR2C plasmid and plated as described in 2.2.4. A single colony was used to inoculate 2xYT media (16 g/l tryptone, 10 g/l yeast extract broth and 5 g/l NaCl) (2 ml) supplemented with thiamine (1 μg/ml) and ampicillin (100 μg/ml). The culture was grown for 3-5 h until saturation at 37°C with vigorous shaking. An aliquot (100 μl) of the culture was used to inoculate 2xYT media (10 ml). Helper phage M13KO7 was added (4 x 10⁸ pfu/ml) and the culture incubated for 60 min at 37°C with vigorous shaking. Kanamycin (70 μg/ml) was then added and the culture incubated overnight at 37°C with vigorous shaking. The overnight culture (10 ml) was aliquotted into sterile microfuge tubes and centrifuged at 12000 g for 5 min at 4°C. The supernatant was transferred to a fresh microfuge tube and centrifuged again at 12000 g for 5 min at 4°C, this was repeated until no pellet was visible (~2-3 times). The supernatants were mixed with 20% (w/v) PEG in NaCl, 2.5 M (200 μl) and incubated at room temperature for 15 min, followed by centrifugation at 12000 g for 10 min at 4°C. The pellets were resuspended in TE buffer, pH 8.0 (100 μl). The solution was extracted with phenol (50 μl)
followed by extraction with chloroform (50 µl). The upper (aqueous) phase was mixed with a solution of absolute ethanol: sodium acetate, 3 M, pH 5.2, 25:1 (300 µl) and incubated for 30 min at -80°C. The ssDNA was pelleted by centrifugation at 12000 g for 10 min at 4°C. The pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE buffer, pH 8.0 (10 µl). The DNA concentration was determined by flat-bed agarose gel electrophoresis as described in 2.2.7.

3.2.4 Transformation of Competent Cells with M13mp18 DNA

The transformation with M13mp18 DNA utilized the blue/white selection procedure to determine the presence of recombinant constructs. Competent NM522 cells were prepared as described 2.2.3. A frozen aliquot (100 µl) of competent NM522 cells was thawed and transferred to a 15 ml polypropylene tube. The M13 DNA (2-5 µl, 20-50 ng DNA) solution was added and mixed gently. The mixture was incubated on ice for 40 min. The cells were heat-shocked by incubating at 42°C for 45 sec in a waterbath, followed by incubation on ice for 5 min. The tubes were allowed to reach room temperature and mixed with a solution of lawn cells (200 µl), IPTG (40 µl of 100 mM stock) and X-gal (40 µl of 2% (w/v) stock). NM522 lawn cells were prepared from a 1:100 dilution of overnight NM522 culture in fresh 2xYT media and grown for 2-3 h when the optical density at λ = 600 nm was 0.3.

Molten H Top agar (10 g/l tryptone, 8 g/l NaCl and 8 g/l agar) (3-4 ml) was added to the transformed cells, mixed by rolling and immediately poured onto an LB-agar plate (LB media + 1.5% (w/v) agar). When the agar had set the plates were inverted and incubated at 37°C for 16-18 h. Blue coloured plaques indicated intact M13mp18 vector DNA; white plaques indicated recombinant constructs of M13mp18NR2C DNA.
CHAPTER 3

3.2.5 Preparation of M13mp18 Replicative Form (RF) DNA

An overnight culture of NM522 cells (2.5 ml) was mixed gently with phage stock (0.5 ml) or inoculated with a fresh phage plaque and incubated for 5 min at room temperature. This was diluted 100:1 with fresh, prewarmed to 37°C 2xYT media and incubated for 5 h at 37°C with shaking at 300 rpm.

For small scale preparation of M13mp18 RF DNA the protocol then followed the small scale plasmid DNA preparation as described in 2.2.5.1.

For large scale preparation of M13mp18 RF DNA the cells were harvested by centrifugation at 20000 g for 15 min at 4°C. The supernatant was discarded and the pellet resuspended by pipetting in ice-cold STE buffer (10 mM Tris-Cl, pH 8.0, 0.1 M NaCl and 1 mM EDTA) (50 ml). The cells were recovered by centrifugation at 20000 g for 15 min at 4°C and the supernatant discarded. The pellet was resuspended in P1 buffer. The large scale preparation of M13mp18 RF DNA then followed the QIAGEN™ plasmid maxi-kit protocol utilizing QIAGEN™ 500 tips as described in 2.2.5.3.

3.2.6 Preparation of M13mp18 ssDNA

Competent NM522 cells were transformed with M13mp18NR2C construct and plated as described 3.2.4. An overnight culture of NM522 cells was diluted 1:50 in fresh 2xYT media. The diluted overnight culture (2 ml) was inoculated with a phage plaque and grown for 4-5 h at 37°C with vigorous shaking. The culture (2 ml) was centrifuged at 12000 g for 5 min at room temperature. The supernatants were transferred to fresh microfuge tubes and recentrifuged at 12000 g for 5 min at room temperature. Phage stocks were prepared from 0.5 ml of the supernatant, the remainder was used to prepare ssDNA. The supernatants were mixed with 20% (w/v) PEG in NaCl, 2.5 M and incubated at room temperature for 15 min. The solution was centrifuged at 12000 g for 10 min at 4°C. The supernatant was aspirated and the pellet resuspended in TE buffer (100 µl) and extracted with phenol (50 µl) followed by extraction with chloroform (50 µl).
The aqueous phase was mixed with a solution of absolute ethanol: sodium acetate, 3 M pH 5.2, 25:1 (300 μl) and incubated for 30 min at -80°C. The ssDNA was precipitated by centrifugation at 12000 g for 10 min at 4°C. The pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE buffer pH 8.0 (10 μl). The ssDNA concentration was determined by flat-bed agarose gel electrophoresis as described in 2.2.7.

3.2.7 Design of Oligonucleotide Primers

The oligonucleotide primers for the mutagenesis and sequencing of the NR2C cDNA were designed using PCR Plan, part of the PC/GENE Software Package (IntelliGenetics Inc., California, U.S.A.). The melting temperature (Tm) was determined by the method of Rychlik et al. (1990). The acceptable Tm range was ± 15°C, optimal primer length was 20 nucleotides with an the acceptable length being 15-30 nucleotides. The allowed maximal number of identical bases in succession was 5. The final 2 bases of the oligonucleotides 3' end were required to be either G or C. This permitted a 'GC' clamp, which allowed tight annealing between the ss template DNA and oligonucleotide and prevented non-specific binding. The acceptable range for percentage of GC residues within the primer was 40-60%. The maximum number of bases required for primer self-complementarity (primer-primer binding) was 4, as was the maximum allowable stem size in stem-loop formation (primer binds to itself). The maximum allowable percentage of bases complementary to other regions within the template was 70%. All of the following primers have been designed for the NR2C subunit cDNA using PCR Plan.
Chapter 3

Primer to create EcoRV site at D440
Silent point mutation; changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>G</th>
<th>F</th>
<th>C</th>
<th>I</th>
<th>D</th>
<th>I</th>
<th>L</th>
<th>K</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (+)</td>
<td>GGC TTC TGC ATC GAC ATC CTC AAG AAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 3'</td>
<td>CG AAG ACG TAG CTA TAG GAG TTC 5'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

The oligonucleotide is 23 bases in length. The primer is not ideal as it has the capability to bind to self.

Primer to create BglII site at R497 and S498
Silent point mutation; changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>S</th>
<th>L</th>
<th>T</th>
<th>I</th>
<th>N</th>
<th>E</th>
<th>E</th>
<th>R</th>
<th>S</th>
<th>E</th>
<th>I</th>
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</thead>
<tbody>
<tr>
<td>Template (+)</td>
<td>TCC CTC ACC ATC AAT GAA GAG CGC TCA GAG ATT</td>
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</tr>
<tr>
<td>Primer 3'</td>
<td>GG GAG TGG TAG TTA CTT CTC TCT AGA CTC TA 5'</td>
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</table>

The oligonucleotide is 30 bases in length. The primer is not ideal as it has the capability to bind to self.

Primer to remove XhoII site at G777 and 1778
Silent point mutation; changes are in bold.

<table>
<thead>
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<th>L</th>
<th>S</th>
<th>G</th>
<th>I</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>E</th>
<th>K</th>
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</thead>
<tbody>
<tr>
<td>Template (+)</td>
<td>TGG CTC TCA GGG ATC TGC CAT AAC GAG AAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 3'</td>
<td>CC GAG AGT CCA TAT ACG GTA TTG CTC 5'</td>
<td></td>
<td></td>
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</tbody>
</table>

The oligonucleotide is 26 bases in length.

Primer to create mutant S583A
Changes are in bold.

<table>
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<tr>
<th>Protein</th>
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<th>I</th>
<th>G</th>
<th>K</th>
<th>S</th>
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<th>W</th>
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<tbody>
<tr>
<td>Template (+)</td>
<td>ACC ATT GGC AAG TCC GTG TGG TTG CTG TGG</td>
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</tr>
<tr>
<td>Primer 3'</td>
<td>GG TAA CCG TTC CGG CAC ACC AAC GA 5'</td>
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<td></td>
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</tr>
</tbody>
</table>

The oligonucleotide is 25 bases in length.
CHAPTER 3

Primer to create mutant V584I
Changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>T</th>
<th>I</th>
<th>G</th>
<th>K</th>
<th>S</th>
<th>V</th>
<th>W</th>
<th>L</th>
<th>L</th>
</tr>
</thead>
</table>

Template (+)  ACC ATT GGC AAG TCC GTG TGG TTG CTG
Primer 3'  GG TAA CCG TTC AGG **TAA** ACC AAC 5'
The oligonucleotide is 23 bases in length.

Primer to create mutant A589G
Changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>W</th>
<th>L</th>
<th>L</th>
<th>W</th>
<th>A</th>
<th>L</th>
<th>V</th>
<th>F</th>
<th>N</th>
</tr>
</thead>
</table>

Template (+)  TGG TTG CTG TGG GCA CTG GTC TTC AAC
Primer 3'  CC AAC GAC ACC **CCT** GAC CAG AA 5'
The oligonucleotide is 22 bases in length.

Primer to create mutant SV583-584AI
Changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>T</th>
<th>I</th>
<th>G</th>
<th>K</th>
<th>S</th>
<th>V</th>
<th>W</th>
<th>L</th>
<th>L</th>
</tr>
</thead>
</table>

Template (+)  ACC ATT GGC AAG TCC GTG TGG TTG CTG
Primer 3'  GG TAA CCG TTC **CGG** **TAA** ACC AAC GA 5'
The oligonucleotide is 25 bases in length.
CHAPTER 3

Primer to create mutant E599Q

Changes are in bold.

<table>
<thead>
<tr>
<th>Protein</th>
<th>V F N N S V P I E N P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template(+)</td>
<td>G GTC TTC AAC AAC TCT GTT CCC ATC GAG AAC CCC</td>
</tr>
<tr>
<td>Primer 3'</td>
<td>C CAG AAG TTG TTG AGA CAA GGG TAG GTC TTG G</td>
</tr>
</tbody>
</table>

The oligonucleotide is 32 bases in length. The primer is not ideal, it is longer than optimal.

Sequencing primer

Used for sequencing ssDNA, primes sequencing of the (+) strand.

| Primer 3' | GGAGCGATCGATGTGCCGTTA 5' |

The oligonucleotide is 22 bases in length. The primer is not ideal as it has the capability to bind to self.

3.2.8 Purification of Oligonucleotide Primers

Oligonucleotides longer than 20 bp were purified by Biospin 30 columns as recommended by the manufacturer. The excess buffer was drained from the columns by gravity. Buffer exchange was achieved by adding TE buffer, pH 8.0 (300 µl) and draining by gravity. Excess buffer was removed by centrifugation at 1100 g for 2 min at 4°C. The oligonucleotide (100 µl) was carefully applied to the centre of the column and allowed to drain into the column. The column was connected to a collecting tube and centrifuged at 1100 g for 4 min at 4°C. The concentration of the purified oligonucleotide was determined spectrophotometrically as described 2.2.6.

3.2.9 Site-Directed Mutagenesis Reaction

The procedure for each step was carried out as recommended by the Sculptor™ in vitro mutagenesis system protocols. The complete mutagenesis
protocol is depicted in Figure 3.1.1. Any modification of the following method will be described in the appropriate results section. All incubations were performed using the RoboCycler™ 40 (Stratagene, La Jolla, CA, U.S.A.)

3.2.9.1 Phosphorylation of oligonucleotide

The concentration of the purified oligonucleotide was adjusted to 0.025 OD\(^{\lambda=260}\) units/ml per base (equivalent to 1.6 pmol/ml). The oligonucleotide (30 µl) was mixed with 10x kinase buffer (3 µl) and T4 polynucleotide kinase (2 units, 2 µl). The reaction was incubated at 37°C for 15 min followed by heat inactivation at 70°C for 10 min. The phosphorylated oligonucleotide was used immediately or stored at -20°C.

3.2.9.2 Annealing of oligonucleotide

The ss template DNA (2 µg), prepared as described in 3.2.3 or 3.2.6, was mixed with the phosphorylated mutagenic oligonucleotide (1 µl), prepared as above. To this was added Buffer A (1.4 M MOPS, pH 8.0 and 1.4 M NaCl) (1 µl) and water to the volume of 10 µl. The solution was mixed by gentle flicking and incubated at 70°C for 3 min followed by 30 min at 37°C. The tube was briefly centrifuged, 10 sec at 12000 g at room temperature, to collect the condensate and placed on ice.

3.2.9.3 Extension and ligation of mutant strand

To the annealing reaction (from 3.2.9.2) was added dNTP mix A (1.01 mM dATP, dCTP\(\alpha\)S, dGTP, dTTP, 2.02 mM ATP and 20 mM MgCl\(_2\)) (10 µl) T4 DNA ligase (2.5 units, 1 µl) and T7 DNA polymerase (1µl, 0.8 units). The reaction solution was mixed by gentle flicking and briefly centrifuging at 12000 g for 10 sec at room temperature. The reaction was incubated for 10 min at room temperature, followed by 30 min at 37°C, then heat inactivated at 70°C for 15 min. Alternatively, the T7 polymerase was replaced by Klenow DNA polymerase
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(1 μl, 4 units), the remainder of the reaction mix was unchanged. In this case the reaction was incubated for 16 h at 16°C followed by heat inactivation at 70°C for 15 min. In either situation, a sample (1 μl) was removed and stored at -20°C for analysis by flat-bed agarose gel electrophoresis as described 2.2.7.

3.2.9.4 Removal of ss (non-mutant) DNA

To the extension reaction (from 3.2.9.3) was added Buffer B (70 mM Tris-HCl, pH 8.0, 10 mM MgCl₂ and 45 mM NaCl) (50 μl) and T5 exonuclease (2 μl, 2000 units). The reaction was mixed and incubated for 30 min at 37°C followed by heat inactivation at 70°C for 20 min. A sample (5 μl) was removed and stored at -20°C for analysis by flat bed agarose gel electrophoresis as described 2.2.7.

3.2.9.5 Nicking of the non-mutant strand

To the T5 exonuclease digestion (from 3.2.9.4) was added Buffer C (700 mM Tris-HCl, pH 8.0, 350 μM EDTA and 20 mM DTT) (5 μl) and restriction enzyme Nci I (1 μl, 5 units). The solution was mixed and incubated for 90 min at 37°C. A sample (10 μl) was removed and stored at -20°C for analysis by flat-bed agarose gel electrophoresis as described 2.2.7.

3.2.9.6 Digestion of the non-mutant strand

To the nicking reaction (from 3.2.9.5) was added Buffer D (250 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 500 μM EDTA) (20μl) and Exonuclease III (1 μl, 160 units). The reaction was mixed and incubated for 30 min at 37°C followed by heat inactivation at 70°C for 15 min. A sample (10 μl) was removed and stored at -20°C for analysis by flat-bed agarose gel electrophoresis as described 2.2.7.
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3.2.9.7 Repolymerization of the gapped DNA

To the exonuclease III digestion (from 3.2.9.6) was added dNTP mix B (1.25 mM dATP, dCTP, dGTP, dTTP, 2.5 mM ATP and 25 mM MgCl₂) (20 µl) and DNA polymerase I (1 µl, 3.5 units) and T4 DNA ligase (1 µl, 2.5 units). The solution was mixed and incubated for 60 min at 37°C. A sample (15 µl) was removed and stored at -20°C for analysis by flat-bed agarose gel electrophoresis as described 2.2.7.

3.2.9.8 Purification of DNA

The dsDNA from the final mutagenesis step was purified using QIAEX DNA resin as described in 2.2.9. This removed the enzymes and salts left from the mutagenesis reactions and concentrated the DNA solution for increased efficiency of transformation.

3.2.9.9 Transformation of competent cells

Competent TG1 E. coli. were used for the transformation as they have a higher transformation efficiency with phosphorothioate DNA than other similar strains. The transformation of competent cells with mutants in plasmid vectors was as described in 2.2.4. If the mutations were carried out with an M13 vector, the transformation was as described in 3.2.4. The plates were inverted and incubated overnight at 37°C. Colonies or plaques were analysed by dideoxy sequencing.

The control mutagenesis reaction using a supplied ssM13 template and supplied phosphorylated oligonucleotide corrects for a point mutation within the β-galactosidase gene which creates a stop codon. The efficiency of mutagenesis can be determined by blue/white screening as described in 3.2.4. Positive mutations give blue plaques; plaques generated by the template DNA are white.
3.2.10 Dideoxynucleotide Sequencing of DNA

The sequencing reactions were carried out using the Sequenase 2.0 kit according to the manufacturers recommendations. The denaturing sequencing gels were prepared and poured as recommended by the manufacturer of the gel apparatus.

3.2.10.1 Sequencing reactions

A single annealing reaction was carried out for each set of four termination reactions. The procedure was the same for both ss plasmid DNA and ss M13 DNA, which were prepared as described in 3.2.3 and 3.2.6 respectively. In a thin-walled microfuge tube ss template DNA (3-5 µg, 2-7 µl) was mixed with 5x Reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl) (2 µl), sequencing primer (1 µl, 1 pmol/µl) and the total volume made up to 10 µl with water. Annealing was carried out by incubation at 65°C for 2 min followed by cooling slowly to <35°C over 30 min. The mixture was briefly centrifuged for 10 sec at 12000 g at room temperature and placed on ice. During the cooling step each Termination Mixture (80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 50 mM NaCl and 8 µM of either ddGTP or ddATP or ddCTP or ddTTP) (2.5 µl) was placed in labelled, thin-walled microfuge tubes. These were kept at room temperature. Prior to setting up the labelling reaction, the labelling mix (7.5 µM dGTP, 7.5 µM dCTP and 7.5 µM dTTP) was diluted 1:5 with water and the Sequanase polymerase was diluted 1:8 in ice-cold enzyme dilution buffer (10 mM Tris-HCl, pH 7.5, 5 mM DTT and 0.5 mg/ml BSA). For the labelling reaction; DTT, 0.1 M (1 µl), diluted labelling mix (2 µl), [³⁵S]dATP (0.5 µl) and diluted Sequanase polymerase (2 µl) were added to the ice-cold annealed DNA mixture. The labelling reaction was mixed and incubated at room temperature for 2-5 min. During this incubation the termination tubes were prewarmed at 37°C. After the incubation, the labelling reaction was aliquotted (3.5 µl) into the termination tubes and incubated at 37°C for 5 min. The
termination reactions were stopped by the addition of Stop Solution (95% (w/v) formamide, 20 mM EDTA, 0.05% (v/v) bromophenol blue and 0.05% (v/v) xylene cyanole FF) (4 µl) and the samples stored on ice. The samples were heated at 75°C for 2 min immediately prior to loading onto the sequencing gel.

### 3.2.10.2 Denaturing gel electrophoresis

Denaturing gel electrophoresis was carried out using Bio-Rad Sequi-Gen™ cell (size 21 x 50 cm), machined-wedge, gradient spacers (thickness 0.25-0.75 mm) and a saw-tooth comb (well-size 2.5 µl). Both plates were thoroughly cleaned with ethanol and acetone, the inner plate/chamber was then siliconized. The gel (6% (w/v) acrylamide/bis-acrylamide, 7 M urea in 1x TBE buffer) was poured 20 h prior to use and the comb inserted. The gel was left to polymerize overnight. Prior to loading the sequencing samples the gel was pre-run for 2 h at 45°C (40 W) with 1 x TBE electrophoresis buffer. The sequencing samples were loaded (2 µl) using an Eppendorf digital ultra-micro pipette and GELoader capillary tips. The gel was run at 50°C (~50 W) until the bromophenol blue band had electrophoresed from the gel. If extended sequencing with 2 loadings was carried out, the first loading was run until the xylene cyanole FF reached the bottom of the gel. The gel was loaded for a second time and run until the bromophenol blue had reached the bottom of the gel. After electrophoresis the Sequi-Gen cell was carefully prised apart, with the gel remaining on the outer plate. The gel and plate were immersed in fixing solution (5% (v/v) acetic acid and 15% (v/v) methanol) and soaked for 60 min at room temperature to remove the urea. The gel was transferred to 3MM filter paper, dried at 80°C for 2 h, placed in a cassette and exposed to film (Kodak X-OMAT AR-5) for 24-48 h. The film was developed in Kodak D-19 developer for 60 sec and fixed for 2 min in Kodak unifix.
3.2.11 CytoTox 96™ Non-Radioactive Cytotoxicity Assay

The CytoTox 96™ assay quantitatively measures the release of lactate dehydrogenase (LDH) from lysed cells. The manufacturer's protocol was adapted for the measurement of LDH release from transfected HEK 293 cells. The HEK 293 cells were transfected as described 2.2.17. 40 h following transfection, an aliquot (0.5 ml) of media was removed and placed in a microfuge tube. The sample was centrifuged at 12000 g for 5 min at 4°C and the supernatant removed to a fresh microfuge tube. The experimental sample was diluted 1:5 in DMEM/F12 media and aliquots (50 μl) were transferred in triplicate to a 96 well plate. The total LDH release was obtained by freeze/thaw lysis of the flask of cells. The flasks were incubated at -70°C for 30 min followed by incubation at 37°C for 15 min. Samples were taken and processed as above. However, the total samples were diluted 1:20 in DMEM/F12 media. Substrate mix (50 μl) was added to all the sample wells, the plate was covered with aluminium foil and incubated for 30 min at room temperature. Stop solution (50 μl) was added to each sample. The absorbance at λ = 490 nm was measured with a Dynatech minireader; the 'blank' was DMEM/F12 media (50 μl) + substrate mix (50 μl) + stop solution (50 μl). The percentage of cytotoxicity was calculated using the formula:

\[
\% \text{ CYTOTOXICITY} = \frac{\text{OD for Experimental LDH release}}{\text{OD for Total LDH release}} \times 100
\]
3.3 RESULTS

3.3.1 Oligonucleotide-directed mutagenesis of the NR2C subunit

Most methods for oligonucleotide-directed mutagenesis, including the Sculptor™ system, require ssDNA. This can be obtained by subcloning the DNA of interest into an M13 vector or into a plasmid with a ssDNA phage origin of replication, such as f1 ori. It is preferable that the fragment of target DNA to be used in the mutagenesis reaction be as small as possible. This should reduce the possibility of non-specific hybridization of the mutagenic oligonucleotide. Moreover, the sequencing of the DNA following the mutagenesis reaction will take less time. The DNA is sequenced following the mutagenesis reaction to confirm the desired mutation is present and no further changes have been made to the DNA sequence. To achieve this criteria, the first part of the mutagenesis strategy was to find or create appropriate restriction sites within the NR2C cDNA sequence to allow the subcloning of a fragment of the NR2C clone.

3.3.1.1 Strategy 1: Creation of a restriction site in NR2C cDNA

The fragment of NR2C DNA to be used in the mutagenesis reaction had to excised from the full cDNA sequence such that it was possible to subclone it back into the same site once mutated, as the mutated NR2C polypeptide was to be expressed in HEK 293 cells. Therefore, unique restriction sites within the pCISNR2C construct were needed. These sites must also be present in a vector containing an origin for ssDNA production. Analysis of the NR2C cDNA sequence showed the presence of a unique NotI site downstream from the M2 region. Further investigation showed a potential EcoRV site upstream from the M2 region, the relationship of these sites to the M2 region is shown schematically in Figure 3.3.1. The EcoRV site could be created by a silent, single-point mutation, i.e. the amino acid encoded for by the codon would not be changed. Restriction digest at the NotI site and created EcoRV site would
generate a fragment of DNA ~1250 bp in length. However, a preliminary restriction enzyme digestion of the pCISNR2C construct (results not shown) demonstrated the presence of an EcoRV site in the 3' untranslated region, see Figure 3.3.1. This meant that the potential EcoRV site was no longer unique, so could not be used for the subcloning of the NR2C fragment. However, the NotI site was still available. A second restriction site was needed approximately 1000-1500 bp upstream from this site. Sequence analysis of the NR2C clone indicated a BglII restriction site could be created by silent point mutations. The BglII and NotI sites would allow the extraction of a DNA fragment of ~1100 bp. A schematic of the NR2C clone indicating these sites is shown in Figure 3.3.2. A preliminary restriction digest showed that neither of these restriction enzyme sites were found in the untranslated region (results not shown). The pBKSII+ vector (Figure 2.3.4) was chosen for the subcloning of the fragment and subsequent mutagenesis reactions. The pBKSII+ vector does not contain a BglII restriction site. However, the BamHI restriction site has compatible ends to BglII. Following ligation the correct ends could be regenerated by cutting with XhoII. The unfortunate presence of an XhoII site, in the DNA fragment to be excised, could be removed by silently mutating the restriction site during the initial mutagenesis reaction. Both of these initial mutagenesis reactions could be screened by restriction digestion, as opposed to sequencing.

The mutagenesis reaction was carried out using the mutagenic oligonucleotide to create a BglII site (see 3.2.7) and ss pCISNR2C DNA as described in detail in 3.2.9.1-11. The control mutagenesis reaction using the 'white' control template and the 'blue' control oligonucleotide, supplied by the manufacturer showed over 95% efficiency. However, no colonies were seen following transformation with pCISNR2C mutagenesis reaction DNA. Each stage of the mutagenesis reaction was analysed by taking a small sample at each step of the reaction, as schematically represented in Figure 3.3.6. The blue/white control mutagenesis reaction had DNA in all the samples and showed the
appropriate pattern of bands when analysed by flat-bed agarose gel electrophoresis, as shown in Figure 3.3.7. The pCISNR2C mutagenesis reaction with the BgIII mutagenic oligonucleotide, showed DNA in sample 1 of the appropriate pattern, but no DNA in samples 2-5. This indicated that the DNA was destroyed in step 3, the T5 exonuclease digestion. This implied that the dsDNA resulting from step 2, the extension and ligation of the mutant strand, was not completely dsDNA. This could result from poor oligonucleotide-template annealing. This was checked by sequencing with the oligonucleotide and template, which was successful. The annealing step of the mutagenesis protocol (see 3.2.9.2) was modified to incubation at 70°C for 3 min, followed by transfer to a heat-block at 55°C which was allowed to cool to room temperature. Modification of the extension and ligation step (see 3.2.9.3) by extending the incubation at room temperature to 30 min was tried, as was shortening the time of the T5 exonuclease incubation. No modification altered the outcome of the pCISNR2C mutagenesis reaction, thus at no point were colonies seen following transformation, nor was any DNA seen in the samples 2-5 taken for analysis. It was concluded that the size of the pCISNR2C construct, 9.1 kb, was too large for the series of mutagenesis reactions and enzymes to work efficiently. The Sculptor™ in vitro mutagenesis system had been characterized for M13 vectors, and was less efficient with plasmid vectors (pers. comm. Amersham Ltd.). Therefore a new strategy was devised.

3.3.1.2 Strategy 2: Mutagenesis of pBKSII+NR2C

Following the failure to create a new restriction site within the pCISNR2C construct (see above) a new strategy was employed. It was thought that the pCISNR2C construct was too large for efficient mutagenesis, especially the T7 DNA polymerase extension step. Therefore, the NR2C clone would be mutated using the pBKSII+ vector. The pBKSII+NR2C construct was 7.36 kb (Figure 2.3.3), compared to the pCISNR2C construct of 9.1 kb (Figure 2.3.6). No attempt was made to create restriction sites within the NR2C clone. The M2
region mutagenesis reactions using the oligonucleotides, S583A, V584I and A589G (see 3.2.7) were carried out on the complete NR2C clone in the pBKSII+ vector. The mutagenesis reactions were performed as described in 3.2.9.1-9 with some alterations, which were as follows. The annealing step of the mutagenesis protocol (see 3.2.9.2) was modified to an incubation at 70°C for 3 min, followed by transfer to a heat-block at 55°C which was then allowed to cool to room temperature. The extension and ligation step (see 3.2.9.3) was changed to a room temperature incubation of 30 min, followed by a 37°C incubation of 30 min and an incubation at 70°C for 15 min. The TG1 transformation was carried out as described in 3.2.9.9. Colonies were seen after transformation for each mutagenesis reaction. Table 3.3.1. The control blue/white mutagenesis reaction showed over 95% efficiency. Single colonies were amplified and ssDNA prepared. The DNA was analysed by flat-bed agarose gel electrophoresis and putative mutants screened for by dideoxy nucleotide sequencing.

3.3.1.2.1 Dideoxy nucleotide sequencing of putative mutants

For dideoxy nucleotide sequencing, the ssDNA was prepared as described in 3.2.3. This DNA was initially analysed by flat-bed agarose gel electrophoresis to determine the concentration of the DNA samples. All of the samples ran at the appropriate size for ss pBSKII+NR2C DNA.

The dideoxy nucleotide sequencing was carried out using a primer which annealed approximately 100 bases upstream of the M2 region of the NR2C clone (see 3.2.7). The sequencing reactions were carried out as described in 3.2.10.1. The results are summarized in Table 3.3.1. In the first instance the potential S583A mutants were analysed. Of the 30 colonies from the putative S583A mutant plate 15 were picked for ssDNA preparation and dideoxy nucleotide sequencing. Sequencing reactions were analysed by denaturing gel electrophoresis and film exposures made as described in 3.2.10.2. All of the pBSKII+NR2C samples gave very faint sequence bands on the film. These
remained so despite extended exposures and the use of fresh [α-35S]-dATP for the sequencing reactions. One sequence was readable for the appropriate region, but it was not mutated. The other 14 sequencing reactions were not readable. For the putative V584I and A589G plates, 8 and 6 colonies were picked respectively. None of the putative V584I mutant samples gave a readable sequence following dideoxy nucleotide sequencing and denaturing gel electrophoresis. The putative A589G mutant samples generated 2 readable, non-mutant sequences and 4 unreadable sequences, see Table 3.3.1. Modification of the sequencing reaction protocol did not improve the sequences. However, the control template and primer supplied with the Sequanase™ 2.0 DNA Sequencing kit gave a clear, readable sequence.

3.3.1.3 Strategy 3: Mutagenesis of M13mp18NR2C

Following the unsuccessful attempts to mutate the NR2C clone using plasmid vectors, a new strategy using an M13 vector was devised. The Sculptor™ in vitro mutagenesis system was optimized for use with M13 vectors (pers. comm., Amersham Ltd.). Previous strategies to subclone a fragment of the NR2C were unsuccessful. Therefore, the complete NR2C clone was subcloned from pCISNR2C into M13mp18, Figure 3.3.3. The construct M13mp18NR2C, see Figure 3.3.4, was used for the mutagenesis reactions. No further attempts to create restriction sites were made, and the mutations of interest (see Figure 3.1.2), in the M2 region, were carried out on the full construct.

3.3.1.3.1 Subcloning of the NR2C cDNA into the M13mp18 vector

The NR2C clone (4.4 kb) was excised from the construct pCISNR2C (Figure 2.3.6) and cloned into the EcoRI site of the M13mp18 vector (Figure 3.3.3). The cloning was non-directional and the orientation of the NR2C clone in the resulting M13mp18NR2C construct (Figure 3.3.4) was checked by restriction digestion.
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The M13mp18 vector was linearized with EcoRI enzyme and the compatible termini were dephosphorylated as described in 2.2.12. The linearized, dephosphorylated DNA was purified using the QIAEX resin (2.2.9). The pCISNR2C construct was digested with EcoRI, to excise the NR2C clone and Scal to destroy the pCIS vector. The resulting fragments (of 4.4 kb, 2.7 kb and 2.0 kb) were separated by flat-bed agarose gel electrophoresis (2.2.7). The 4.4 kb fragment was excised from the agarose gel and purified with QIAEX resin (2.2.11). The ligation reaction was set up as described in 2.2.13. Competent E. coli of the NM522 strain were transformed as described in 3.2.4 and recombinant selection utilized the blue/white selection procedure. Possible recombinant plaques were amplified and dsDNA prepared as described in 3.2.5. The DNA samples were analysed by EcoRI and Scal restriction enzyme digestion to confirm the presence of the NR2C clone and to confirm its orientation. The restriction enzyme analysis is shown in Figure 3.3.5. Lanes 3 and 5 show uncut M13mp18 and M13mp18NR2C respectively, lanes 4 and 6 show linear, cut M13mp18 and M13mp18NR2C respectively. In lane 7 the M13mp18NR2C is digested with EcoRI demonstrating the excision of the NR2C clone. Lane 8 demonstrates that the NR2C clone is in the desired sense orientation. Scal digestion of correctly orientated M13mp18NR2C generates 2 fragments of 8.0 kb and 3.5 kb, incorrect orientation would generate 2 fragments of size 10.7 kb and 0.8 kb.

3.3.1.3.2 Mutagenesis reactions

The positions of the desired mutations are shown in Figure 3.1.2. The mutagenesis reactions were carried out with the mutagenic oligonucleotides, S583A, V584I, A589G and E599Q (see 3.2.7) as described in 3.2.9.1-9, with some modifications. These were as follows; the amount of M13mp18NR2C template ssDNA was increased to 3 μg, a second oligonucleotide which annealed to the M13mp18 vector was included. Both of the alterations were due to the large size, 11.7 kb, of the construct. The second oligonucleotide provided a
second starting point for the extension reaction. The extension reaction was carried out using Klenow polymerase for 16 h at 16°C. The SV583-584A1 mutagenesis reaction utilized ssM13mp18NR2C(A589G) for the template. This allowed the creation of the three mutagenic changes in the M2 region. The mutagenesis reaction was carried out as described in 3.2.9.1-9 with the modifications detailed above. For all of the mutagenesis reactions, the transformation utilized blue/white selection. This aided the elimination of M13mp18 revertants which had lost the NR2C clone. Blue plaques were assumed to be revertants and not analysed further. However, plaques which were white could still have lost the NR2C clone. Each stage of the mutagenesis reaction was analysed by taking a small sample at each step of the reaction, as schematically shown in Figure 3.3.6. The result of this analysis for the control mutagenesis reaction is shown in Figure 3.3.7. Lanes 2 and 3 show dsM13mp18 and ssM13mp18 DNA respectively. Lane 4 clearly shows the presence of dsM13mp18 DNA, this is sample 1 from the mutagenesis reaction. Lanes 5 and 6 also show dsM13mp18 DNA in samples 2 and 3. The control mutation supplied with the Sculptor™ in vitro mutagenesis kit showed over 90% efficiency for each experiment carried out. The controls for the mutagenesis reactions indicated a successful experiment. Therefore, single plaques were amplified and ssDNA prepared. The DNA was analysed by flat-bed agarose gel electrophoresis and putative mutants screened by dideoxy nucleotide sequencing.

3.3.1.3.3 Dideoxy nucleotide sequencing of putative mutants

For dideoxy nucleotide sequencing, ssDNA was prepared as described in 3.2.6. The ssDNA was initially analysed by flat-bed agarose gel electrophoresis to check the concentration and size of the DNA, due to the instability of the NR2C clone insert in the M13mp18 vector. Samples of ssDNA which ran differently from known ssM13mp18NR2C on flat-bed agarose gel electrophoresis were not analysed by dideoxy nucleotide sequencing, these samples were
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considered to be M13mp18 vector which had lost the NR2C insert (see Table 3.3.2).

For the initial dideoxy nucleotide sequencing, a primer which annealed approximately 100 bases upstream of the M2 region was used (see 3.2.7). The sequencing reactions were carried out as described in 3.2.10.1. Of the 200 white plaques from the putative S583A mutant plate, 10 were picked for ssDNA preparation and of the 10 ssDNA samples 8 were analysed by dideoxy nucleotide sequencing and denaturing gel electrophoresis. Six samples had the specific mutation in their coding regions, with no other changes in the sequenced region, which was 300-350 bases. Two of these samples were further analysed to confirm the specificity of the mutation. The sequencing reactions were repeated with the sequencing primer and with the EcoRV site primer and the XhoII site primer (see 3.2.7) which annealed 500 bases upstream and 450 bases downstream of the sequencing primer respectively. Double loading of the sequencing gel was used to extend the readable DNA sequence. The sequenced region of approximately 1000-1100 bases did not show any non-desired mutations.

Ten white plaques were picked from the V584I and A589G plates and ssDNA templates (3.2.6) were prepared. Following flat-bed agarose gel electrophoresis, 6 putative V584I samples and 7 putative A589G samples were sequenced using the same sequencing primer as above (3.2.7). All of the sequenced A589G samples and 4 of the 6 V584I samples had the specific mutation with no other changes in the sequenced region. Two of each of these mutations were further checked with the EcoRV site primer and XhoII site primers as described above. The sequenced region was 1000-1100 in total for each of the samples. Only the desired mutations were seen within the sequenced region for any of the samples.

For the putative NR2C(SV583-584AI, A589G) mutation, 30 of the 50 white plaques were picked for ssDNA preparation. After analysis by flat-bed agarose gel electrophoresis, 21 of these samples were sequenced using the
sequencing primer (3.2.7). None of these samples had the specific mutations required for the SV583-584AI mutation, although all retained the A589G mutation of the template.

The first E599Q mutagenesis reaction generated a large number of blue plaques, implying a large number of M13mp18 revertant plaques, see Table 3.3.2. Moreover, the 20 white plaques selected for ssDNA preparation generated ssM13mp18 DNA. None of these samples were sequenced and the mutagenesis reaction was repeated. Following the transformation of the competent cells with the second E599Q mutagenesis reaction, a more appropriate proportion of blue to white plaques was seen, see Table 3.3.2. Ten white plaques were picked for ssDNA preparation, of these, 8 were M13mp18 revertants and 2 were M13mp18NR2C recombinants. The recombinant samples were sequenced, however, neither showed any changes from the wild-type NR2C cDNA in the sequenced region.

Successfully mutated NR2C clones, NR2C(S583A), NR2C(V584I) and NR2C(A589G) were subcloned back into the pCIS mammalian expression vector.

3.3.1.3.4 Subcloning of the mutated NR2C cDNA into the pCIS vector

The mutated NR2C clones (4.4 kb) were excised from the construct M13mp18NR2C (Figure 3.3.4) and cloned into the EcoRI site of the pCIS vector (Figure 2.3.5). The cloning was non-directional and the orientation of the NR2C clone in the resulting pCISNR2C construct (Figure 2.3.6) was checked by restriction digestion.

The pCIS vector was linearized with EcoRI enzyme and the compatible termini were dephosphorylated as described in 2.2.12. The linearized, dephosphorylated DNA was purified using the QIAEX resin (2.2.9). The construct M13mp18NR2C was digested with EcoRI, to excise the NR2C clone. The resulting fragments (of 7.25 kb and 4.4 kb,) were separated by flat-bed
agarose gel electrophoresis (2.2.7). The 4.4 kb fragment was excised from the agarose gel and purified with QIAEX resin (2.2.11). The ligation reaction was set up as described in 2.2.13. Competent \textit{E. coli} of the INV\textalpha strain were transformed as described in 2.2.4. and plated on LB-ampicillin plates. Colonies were amplified overnight and dsDNA prepared as described in 2.2.5.1. The DNA samples were analysed by restriction digestion as described in 2.2.8. Figure 3.3.8 shows the restriction analysis results for pCISNR2C(A589G), lane 3 shows the size of the M13mpl8NR2C construct, lanes 5 and 9 indicates the sizes of the pCIS vector and pCISNR2C construct respectively. Lane 8 shows pCISNR2C digested with \textit{SalI} indicating the correct orientation of the NR2C clone, with fragments of 5.4 kb and 3.7 kb. The same subcloning and restriction analysis results were obtained with the mutants NR2C(S583A) and NR2C(V584I).

3.3.2 Two-point $[^3H]$MK801 binding to HEK 293 cell homogenates following co-transfection with wild-type pCISNR1a and single-point mutated pCISNR2C

The previous chapter described the expression of the NR2C polypeptide in HEK 293 cells and following co-transfection with pCISNR1a, the optimization of $[^3H]$MK801 binding to the recombinant receptor NR1a/NR2C. It was noted that the NR1a/NR2C 1:10 receptor had a low affinity for $[^3H]$MK801 ($K_D = 346 \pm 158$ nM), while the NR1a/NR2A 1:3 receptor had a high affinity ($K_D = 7.0 \pm 2.4$ nM). To investigate further the $[^3H]$MK801 binding site and the requirements for high affinity $[^3H]$MK801 binding a series of mutations in the NR2C subunit were made, as described in 3.3.1.3.2 and 3.3.1.3.3.

HEK 293 cells were co-transfected with wild-type pCISNR1a DNA and one of the mutant pCISNR2C constructs by the Ca$^{2+}$-phosphate method as described in 2.2.17. The DNA ratio used was 1:10 pCISNR1a:pCISNR2C, this ratio had previously been determined as optimal for $[^3H]$MK801 binding to the recombinant, wild-type NR1a/NR2C receptor, see Table 2.3.2. Cells were
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harvested 40 h post-transfection as described in 2.2.19.

The expression of the polypeptides was determined by immunoblotting with subunit-specific antibodies. Figure 3.3.9 shows an immunoblot of HEK 293 cell homogenates which had been separately transfected with wild-type pCISNR2C, pCISNR2C(S583A), pCISNR2C(V584I) or pCISNR2C(A589G) and wild-type NR1a cDNA. The immunoblot was probed with the C-terminal anti-NR2C antibody. The subunit-specific antibody recognized a polypeptide with M, 145000 from all of the transfections, thus demonstrating the presence of a point mutation did not affect the transcription, translation and expression of the NR2C subunit. Incubation of the antibody with the peptide used for immunization prior to immunoblotting blocked the signals, see Figure 3.3.9. The lower bands, of less than 97 kDa, also detected by the subunit-specific antibody are presumably proteolytic fragments, as these signals are also blocked by pre-incubation of the antibody with the peptide used for immunization. The different intensities of the signals in Figure 3.3.9 were due to the varying amounts protein loaded per lane, rather than differences in level of expression of the subunits. All of the 145000 kDa bands showed some evidence of doublets, this may be due to the presence of glycosylated and non-glycosylated NR2C polypeptide. A change in the molecular weight of the NR2C subunit following treatment with N-glycanase was shown by Chazot et al. (1994). The presence of doublet bands was seen previously, when the wild-type NR2C subunit was co-expressed with NR1a, Figure 2.3.11. However, the non-glycosylated peptide was not seen when the NR2C cDNA was transfected and expressed alone, see Figure 2.3.9.

Two point radioligand binding was carried out on the transfected HEK 293 cell homogenates as described in 2.2.22. The level of [³H]MK801 binding was compared to wild-type NR1a/NR2C receptors and wild-type NR1a/NR2A receptors, see Table 3.3.3. The signal: noise ratio was also determined, see Table 3.3.4. None of the mutant NR1a/NR2C recombinant receptors had [³H]MK801 binding levels greater than the wild-type NR1a/NR2C receptor at either of the
\[^{3}H\]MK801 concentrations used. Moreover, all of the NR1a/NR2C cloned receptors (wild-type and mutant) had significantly lower levels of \[^{3}H\]MK801 binding than the wild-type NR1a/NR2A cloned receptor. The signal:noise ratio of the mutant NR1a/NR2C receptors were similar to the wild-type NR1a/NR2C receptor, especially with the 50 nM \[^{3}H\]MK801 concentration. Interestingly, the mutant receptor NR1a/NR2C(S583A) had significantly lower specific binding at the \[^{3}H\]MK801 concentration of 30 nM as compared to the wild-type NR1a/NR2C receptor. The signal:noise ratio of the NR1a/NR2C(S583A) receptor was also lower than the wild-type NR1a/NR2C receptor at the \[^{3}H\]MK801 concentration of 30 nM. Neither of these differences were seen at the higher \[^{3}H\]MK801 concentration of 50 nM. Attempts to determine the \[^{3}H\]MK801 affinity of the mutant NR1a/NR2C receptors by either saturation binding or displacement assays were unsuccessful due to the low levels of binding and low signal:noise ratio. For all the NR1a/NR2C type receptors the signal:noise ratio was significantly lower than that seen for the NR1a/NR2A receptor, Table 3.3.4.

3.3.3 The effects of mutations on HEK 293 cell survival following cotransfection with the wild-type pCISNR1a and mutant pCISNR2C

Co-transfections with the three cDNAs NR1a, NR2A and NR2C generated a recombinant receptor which was lethal to the HEK 293 cells, see Table 2.3.5. This lethality was also seen with co-expression of the NR1a and NR2A subunits. However, it was not seen with expression of the NR1a/NR2C recombinant receptor (2.3.4.1). The NR2C subunit amino acids were mutated to the equivalent NR2A residues. Therefore, their effects on receptor-mediated cell death were investigated.

Following co-transfection with the wild-type NR1a cDNA and one of the mutant NR2C cDNAs, with a 1:10 DNA ratio, the HEK 293 cells were cultured
for 40 h post-transfection. The percentage cell death was determined by LDH release using the CytoTox 96™ cytotoxicity assay as described 3.2.11. The results are summarized in Table 3.3.5. The results from the mutant receptors were compared to those obtained from wild-type NR1a/NR2C recombinant receptors and the NR1a/NR2A recombinant receptor population. These controls were always transfected and assayed in parallel with the mutant recombinant receptors. Expression of the NR1a/NR2A wild-type receptor gives 100% mortality of transfected cells (Cik et al., 1993). However, approximately only 30% of the HEK 293 cell population is transfected by the Ca\(^{2+}\)-phosphate method. This explains why a 100% LDH release when determining the receptor-mediated cell death is not seen. The presence of the AP5 reduced the NR1a/NR2A receptor mediated cytotoxicity by 75% as previously determined (Cik et al., 1993). Again in accordance with previous studies, expression of the NR1a/NR2C wild-type receptor gave no significant levels of cell death (see Table 2.3.5). The individual point mutations of the NR2C subunit did not significantly alter the level of cell death when mutant recombinant NR1a/NR2C receptors were expressed, as compared to the wild-type recombinant NR1a/NR2C receptor. The low levels of LDH release seen with the expression of NR1a/NR2C (wild-type and mutant) receptors were not significantly different from that seen with untransfected cells. These levels are presumed to be due to normal cell turnover.
Figure 3.3.1 Schematic representation of the NR2C clone showing the NotI, proposed EcoRV and actual EcoRV restriction sites.

The point mutation required to generate the EcoRV site is indicated by the arrow.
Figure 3.3.2

Schematic representation of the NR2C clone showing the *NotI*, *XhoII* and proposed *BglII* restriction sites

The mutations required to generate the *BglII* site are indicated by the arrows.
## Table 3.3.1

Analysis of TG1 colonies following transformation with potentially mutant pBKSII+NR2C DNA

<table>
<thead>
<tr>
<th>Mutagenesis Reaction</th>
<th>Colonies after Transformation</th>
<th>Number of ssDNA Preparations</th>
<th>Results from Sequencing Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2C(S583A)</td>
<td>30</td>
<td>15</td>
<td>1 wild-type</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 unreadable</td>
</tr>
<tr>
<td>NR2C(V584I)</td>
<td>9</td>
<td>8</td>
<td>8 unreadable</td>
</tr>
<tr>
<td>NR2C(A589G)</td>
<td>7</td>
<td>6</td>
<td>2 wild-type</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 unreadable</td>
</tr>
</tbody>
</table>
The vector M13mp18 is derived from M13 phage. The MCS is located within the LacZ gene allowing blue/white selection of the recombinants. The vector can produce dsDNA or ssDNA of the (+) strand.
Figure 3.3.4  Restriction map of the construct M13mp18NR2C

This construct was generated on the subcloning of the NR2C clone from pCIS into the M13mp18 vector (3.3.1.3.1).
Figure 3.3.5  Restriction enzyme analysis of the recombinant M13mp18NR2C

Lanes:
1 and 9 Molecular weight standards
2  Blank
3  M13mp18
4  M13mp18 digested with EcoRI
5  M13mp18NR2C
6  M13mp18NR2C digested with BglII
7  M13mp18NR2C digested with EcoRI
8  M13mp18NR2C digested with Sall
Figure 3.3.6 Schematic representation of the analysis of the *in vitro* mutagenesis reaction
**CHAPTER 3**

- **Mutant Oligonucleotide**
- **Recombinant ssDNA Template**
- **Anneal**
- **Extension + Ligation**
- **Removal of ssDNA Template**
- **Nicking Non-Mutant Strand**
- **Gapping Non-Mutant Strand**
- **Repolymerization**
- **Transformation**
Figure 3.3.7  Typical analysis of the *in vitro* mutagenesis reaction

Lanes:
1 and 10 Molecular weight standards
2  dsM13mp18
3  ssM13mp18
4  Sample 1
5  Sample 2
6  Sample 3
7  Sample 4
8  Sample 5
9  dsDNA following QIAEX purification
CHAPTER 3

<table>
<thead>
<tr>
<th>Mutagenesis Reaction</th>
<th>Plaques after Transformation</th>
<th>ssDNA Preparations</th>
<th>Results from Sequencing Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blue</td>
<td>White</td>
<td>8 recombinant</td>
</tr>
<tr>
<td>NR2C(S583A)</td>
<td>0</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 M13mp18</td>
</tr>
<tr>
<td>NR2C(V584I)</td>
<td>6</td>
<td>20</td>
<td>6 recombinant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NR2C(A589G)</td>
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<td>85</td>
<td>7 recombinant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR2C(E589G) (SV583-584AI, A589G)</td>
<td>9</td>
<td>50</td>
<td>21 recombinant</td>
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<tr>
<td>NR2C(E599Q) (1st reaction)</td>
<td>47</td>
<td>60</td>
<td>20 M13mp18</td>
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<tr>
<td>NR2C(E599Q) (2nd reaction)</td>
<td>7</td>
<td>60</td>
<td>2 recombinant</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Table 3.3.2 Analysis of TG1 plaques following transformation with potentially mutant M13mp18NR2C DNAs

The ss template DNA was previously mutated M13mp18NR2C(A589G)

Analysis of the transformants utilized blue/white selection as described in 3.2.9.9. Only white plaques were analysed further.
Figure 3.3.8  Restriction analysis of mutant pCISNR2C

Lanes:
1 and 10 Molecular weight standards
2  M13mp18
3  M13mp18NR2C digested with BglII
4  pCIS
5  pCIS digested with Salt
6  pCISNR2C
7  pCISNR2C digested with EcoRI
8  pCISNR2C digested with Salt
9  pCISNR2C digested with Scal
Cell homogenates were prepared from HEK 293 cells transfected with mutant pCISNR2C and wild-type pCISNR2C. The position of the molecular weight markers (kDa) are shown on the left.

Lanes:
1 and 6 Untransfected HEK 293 cells
2 and 7 NR2C(S583A)
3 and 8 NR2C(V584I)
4 and 9 NR2C(A589G)
5 and 10 Wild-type NR2C
1-10 Probed with anti-NR2C (1208-1218)
6-10 The antibody was pre-incubated overnight at 4°C with the peptide that was used for antibody production.
### Table 3.3.3 Effect of mutations on the NR2C subunit on $[^3H]$MK801 binding following co-transfection with wild-type pCISNR1a and mutant pCISNR2C

HEK 293 cells were co-transfected with NR1a and wild-type NR2A (with a ratio 1:3) or with wild-type NR2C or mutant NR2C cDNAs (with a ratio of 1:10) and cultured for 40 h. Following harvesting and membrane preparation, the recombinant receptors were assayed using two $[^3H]$MK801 concentrations and 10 μM (+)MK801 to determine non-specific binding as described in 2.2.22. All values are the mean ± S.D. for n = 4 determinations from separate transfections.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ratio of Total/ Non-specific Binding</th>
<th>Concentrations of $[^3H]MK801$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 nM</td>
</tr>
<tr>
<td>NR1a/NR2A</td>
<td>1.69 ± 0.05</td>
<td>1.74 ± 0.04</td>
</tr>
<tr>
<td>NR1a/NR2C</td>
<td>1.26 ± 0.06</td>
<td>1.19 ± 0.04</td>
</tr>
<tr>
<td>NR1a/NR2C(S583A)</td>
<td>1.09 ± 0.04</td>
<td>1.19 ± 0.06</td>
</tr>
<tr>
<td>NR1a/NR2C(V584I)</td>
<td>1.12 ± 0.10</td>
<td>1.24 ± 0.04</td>
</tr>
<tr>
<td>NR1a/NR2C(A589G)</td>
<td>1.25 ± 0.08</td>
<td>1.20 ± 0.11</td>
</tr>
</tbody>
</table>

**Table 3.3.4** The signal:noise ratio for $[^3H]MK801$ binding to the wild-type NR1a/NR2A and NR1a/NR2C receptors and the mutant NR1a/NR2C receptors
<table>
<thead>
<tr>
<th>Transfection Sample</th>
<th>% LDH Release</th>
<th>% LDH Release Normalized to NR1a/NR2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1a/NR2A</td>
<td>28.6 ± 3.1</td>
<td>100</td>
</tr>
<tr>
<td>NR1a/NR2A + AP5 (400 μM)</td>
<td>7.0 ± 1.4</td>
<td>24.5</td>
</tr>
<tr>
<td>NR1a/NR2C</td>
<td>0.66 ± 1.1</td>
<td>2.3</td>
</tr>
<tr>
<td>NR1a/NR2C(S583A)</td>
<td>1.3 ± 1.2</td>
<td>4.5</td>
</tr>
<tr>
<td>NR1a/NR2C(I584V)</td>
<td>0.45 ± 1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>NR1a/NR2C(A589G)</td>
<td>1.3 ± 2.0</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 3.3.5 Effect of mutations on the NR2C subunit on HEK 293 cell cytotoxicity following co-transfection with wild-type pCISNR1a and mutant pCISNR2C

HEK 293 cells were co-transfected with the wild-type and mutant cDNAs as shown, using the previously established DNA ratios, 1:3 of NR1a:NR2A and 1:10 of NR1a:NR2C (wild-type and mutant cDNAs). The cells were cultured for 40 h post-transfection. Cell viability was determined for each sample in triplicate, by LDH release using the CytoTox 96™ assay. Values are mean ± S.D. for at least n = 4 determinations from separate transfections.
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3.4 DISCUSSION

In the previous chapter it was shown that cloned NR1a/NR2C receptors had a significantly lower affinity for \(^{[3]H}\)MK801 than recombinant NR1a/NR2A receptors. MK801 has been demonstrated to be a potent NMDA receptor antagonist which selectively binds to open NMDA receptor channels, within the channel pore (Wong et al., 1986; Huettner and Bean, 1988). The M2 segment of the ionotropic glutamate receptor subunits is considered to be the essential component of the ionic pore (see 1.3.3.1; 1.3.3.5; 1.3.5.1). Therefore, the M2 regions of the NR2A and NR2C subunits were compared for potential differences. Sequence comparison of the polypeptides showed only 3 amino acid differences within the M2 region (Figure 3.1.2). There were further amino acid differences immediately outside the M2 region on the C-terminal end. The valine in NR2A (V601) corresponded to an isoleucine in NR2C (I598) and more interestingly, the uncharged glutamine of NR2A (Q602) corresponded to a negatively charged glutamate of NR2C (E599) (Figure 3.1.2). In this study, a selection of mutations were chosen to be introduced into the NR2C subunit. These mutations were S583A, V584I, A589G and SVA-AIG within the M2 region and E599Q outside the M2 region. The rationale for the NR2C(E599Q) mutation followed from a study on the Xenopus nAChR which showed that an equivalently positioned residue determined the alteration in conductance following the developmental change of \(\alpha_2\beta\delta\gamma\) embryonic type nAChRs to \(\alpha_2\beta\delta\varepsilon\) adult type nAChRs (Murray et al., 1995). The \(\gamma\) subunit contained a positively charged lysine, while the \(\varepsilon\) subunit contained a neutral methionine residue. The NR2C(E599Q) mutation would replace a charged glutamate with an uncharged glutamine. However, the accepted topological model for the NMDA receptor subunits changed from one similar to the nAChR receptor, with a transmembrane M2 region, to one containing a pin-loop M2 region, see Figure 1.4. This altered the position of the E599 residue from facing the synaptic cleft to being intracellular.
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To reduce the size of DNA to be subjected to the mutagenesis reaction, the initial strategy was to subclone a fragment of the NR2C clone into a separate vector. However, analysis of the sequence failed to locate appropriate restriction enzyme sites. Therefore, it was attempted to create a new restriction enzyme site by a silent point mutation (3.3.1.1). However, despite modifications of the mutagenesis protocol no colonies were obtained after transformation, nor was DNA detected in the samples taken to monitor the success of the mutagenesis reaction. This could have been due to the size of the pCISNR2C construct and the use of a plasmid to produce the ssDNA, as opposed to M13 (see later). The Sculptor™ in vitro mutagenesis system was optimized for use with an M13 vector (Amersham, pers. comm.). Secondary structure of the ssDNA could also inhibit successful extension in Step 2 (Figure 3.1.1). The presence of any gaps in the newly synthesised strand would allow it to be digested away in Step 5 (Figure 3.1.1).

As the initial strategy was unsuccessful, possibly due to the vector used and the size of the construct, a new strategy was undertaken. The construct pBKSII+NR2C was used for the mutagenesis reaction. This construct was approximately 2.7 kb smaller than pCISNR2C. The change of vector meant it was no longer possible to remove a fragment of the NR2C cDNA. Therefore the mutagenesis reactions were carried out on the complete NR2C clone. Colonies were present following the transformation reaction with DNA from the mutagenesis reactions. However, ssDNA prepared from the putative mutant colonies was not successfully sequenced. Dideoxynucleotide sequencing was carried out with a ssDNA sequencing primer which annealed to the NR2C cDNA approximately 100 bases downstream of the mutated region. Of the sequencing reactions which gave a readable sequence, the bands were very faint and the readable sequence short. The majority of the DNA samples generated no readable sequence. Substitution of the dGTP labelling mix and termination mixtures with the equivalent dITP mixtures did not improve the sequence.
ladders. However, the control template and primer or prepared pCISNR2C ssDNA and sequencing primer gave clear readable sequences after dideoxy sequencing reactions and denaturing gel electrophoresis when carried out in parallel to the putative mutant samples. The inability to sequence the pBKSII+NR2C ssDNA was possibly due to the presence of strong secondary structure. It is of interest to note that the NR2C clone at 4.4 kb is larger than the pBKSII+ plasmid at 2.96 kb. This may allow the formation of more secondary structure within the NR2C cDNA, which is more stably maintained than would be the case if the NR2C clone was in a plasmid of greater size.

As the use of phagemid vectors had been unsuccessful, the NR2C clone was subcloned into the M13mp18 vector. The mutagenesis reactions were carried out as described in 3.2.9.1-9, with the modifications listed in 3.3.1.3.2. The control mutagenesis reaction showed over 90% efficiency for each experiment. Samples of ssDNA prepared from putative mutant plaques (white) were firstly analysed by flat-bed agarose gel electrophoresis. This allowed the detection of M13mp18 revertants which had lost the NR2C clone. Samples which still contained the NR2C clone were then analysed by dideoxy sequencing using the ss sequencing primer (3.2.7). The mutants S583A, V584I and A589G were sequenced through the mutated region, to ensure only the desired changes were present. Further sequencing confirmed that no other changes had been introduced elsewhere. The putative mutant SVA-AIG was also sequenced through the mutated region (21 plaques) but the desired mutations were not present. Flat-bed agarose gel electrophoresis of putative E599Q mutants (20 white plaques) from the initial mutagenesis reaction showed only M13mp18 revertants. There was also a high number of blue M13 plaques present. A second E599Q mutagenesis reaction generated a lower level of blue M13 plaques. However, dideoxynucleotide sequencing of putative E599Q mutants showed no changes to the DNA sequence. The E599Q primer was longer than optimal. However, all the other factors for efficient priming were present (3.2.7).
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The reasons why the triple mutation (SVA-AIG) and the E599Q mutation were not successfully created are not clear. However, mutagenesis of the reverse strand of the NR2C cDNA or mutagenesis by PCR should overcome any problems in further studies.

Following the site-directed mutagenesis reactions, the successfully mutated NR2C clones were subcloned back into the pCIS vector (3.3.1.3.4). The mutants, pCISNR2C(S583A), pCISNR2C(V584I) and pCISNR2C(A589G) were generated and used for transfection of HEK 293 cells. The expression of the mutant NR2C polypeptides was verified by immunoblotting using anti-NR2C antibodies.

Following co-expression of wild-type NR1a and mutant NR2C polypeptides the [$^3$H]MK801 binding was measured using two concentrations of the radioligand, 30 nM and 50 nM. The binding values obtained with the mutant receptors were compared to the values measured from the wild-type NR1a/NR2C and NR1a/NR2A recombinant receptors; these values were always determined in parallel (3.3.2). At neither radioligand concentration, 30 nM or 50 nM of [$^3$H]MK801, was an increase in binding seen for any of the mutant NR1a/NR2C receptors as compared to the wild-type NR1a/NR2C receptor. In fact the binding to the NR1a/NR2C(S583A) mutant receptor was significantly less than the wild-type NR1a/NR2C receptor at a [$^3$H]MK801 radioligand concentration of 30 nM, however, at 50 nM [$^3$H]MK801 the binding between NR1a/NR2C(S583A) and NR1a/NR2C was equivalent. Binding to the wild-type NR1a/NR2A recombinant receptor was significantly higher than all of the mutant NR1a/NR2C receptors at both radioligand concentrations. Attempts to characterize the [$^3$H]MK801 binding affinity of the mutant NR1a/NR2C receptors by displacement assays were unsuccessful due to the high signal: noise ratio. Full saturation binding analysis was not carried out on the mutant receptors. Previous analysis of the wild-type NR1a/NR2C 1:10 recombinant receptor had shown that the specific binding was not saturable at concentrations of [$^3$H]MK801 up to 70 nM (2.3.4.2). The [$^3$H]MK801 radioligand binding data determined for the mutant NR1a/NR2C
receptors by the single-point studies and the unsuccessful displacement assays (results not shown) suggested that the affinity for [³H]MK801 of the mutant NR1a/NR2C receptors was not significantly increased from the wild-type NR1a/NR2C.

Other studies to determine the residues involved in MK801 binding have involved mutating the GluR1 polypeptide, which is weakly sensitive to MK801. The GluR1 subunit residues were mutated to the equivalent NR1 amino acids (Ferrer-Montiel et al., 1995). The presence of the NR1 residues W577 and N598, both located within the M2 region, gave the mutant GluR1(L577W, Q582N) subunit increased sensitivity to MK801. The substitution of a residue in the M3 region generating the mutant GluR1(S610A) also increased the MK801 sensitivity as compared to the wild-type GluR1 subunit. The triple mutation of GluR1(L577W/Q582N/S610A) had an 800-fold increase in sensitivity to MK801 as compared to the wild-type GluR1 subunit (Ferrer-Montiel et al., 1995), thus demonstrating that these residues are important for MK801 binding. However, these residues W577, N598 and A627 (NR1 positions) are conserved across all the NMDA subunits. Therefore they cannot explain the difference in MK801 affinity of the NR1/NR2A and NR1/NR2C recombinant receptors.

Previous studies had shown that transient expression of NR1a/NR2C cloned receptors had no cytotoxic effect upon the HEK 293 cells, unlike expression of NR1a/NR2A receptors (Table 2.3.4). The cytotoxic effect of the expressed NR1a/NR2A receptors is proposed to be due to sustained Ca²⁺-influx through the open pore of the receptor (Cik et al., 1993). The lack of cytotoxicity seen with the recombinant NR1a/NR2C receptor is thought to be due to the greater strength of Ca²⁺ as a permeant blocker of this channel (Burnashev et al., 1995). Therefore, modifying the M2 pore-forming region of the NR2C subunit could in turn alter the cytotoxic properties of the recombinant NR1a/NR2C receptor. LDH release was used to measure the cytotoxic effects of the mutant NR1a/NR2C receptors (3.3.3). The measurements were normalized to the values
determined for HEK 293 cells transfected with wild-type NR1a and NR2A clones. None of the mutated NR2C subunits affected the cell mortality and the values for LDH release following transient co-expression of mutant NR1a/NR2C receptors were not significantly different to the wild-type NR1a/NR2C receptor. Only the NR1a/NR2A receptor gave significantly higher levels of cell death than that seen with untransfected cells. This would suggest that none of the individually mutated residues are directly involved in the permeant Ca\(^{2+}\)-block site of the NR1a/NR2C channel and they do not have a significant influence on the passage of Ca\(^{2+}\) ions. The presence of the triple mutation may have exerted a greater influence on the cytotoxicity of the expressed NR1a/NR2C receptors as this would have generated an M2 region identical to the NR2A subunit. However, this could not be examined.

A report by Raditsch et al. (1993) looking at the block of NMDA receptors by argiotoxin\(_{636}\) (ATX) found that NR1/NR2A receptors expressed in *Xenopus* oocytes were 50-fold more sensitive to ATX than expressed NR1/NR2C receptors. ATX is analogous to MK801 in that both bind within the pore of the NMDA receptor, thereby blocking the channel. The mutations, N595Q in NR2A or N593Q in NR2C (the site corresponds to the Q/R site of the GluR subunits) increased the affinity for ATX for both NR1/NR2A and NR1/NR2C receptors. However, mutating the NR2C M2 region amino acids to the NR2A equivalent residues, i.e. NR2C(SVA-AIG), had no significant effect on the measured ATX affinity for the mutant NR1/NR2C receptor as compared to the wild-type recombinant receptor. This suggests that the subunit-specific differences are not due to the M2 region. The NR2C(E599Q) mutant also had no effect upon the ATX affinity of the NR1/NR2C receptor when co-expressed with the NR1 subunit.

In this study, individually mutating the amino acids in the NR2C subunit M2 region to the equivalent NR2A subunit residues had no detectable effect upon the MK801 affinity or cytotoxicity of the expressed NR1a/NR2C receptors.
The triple mutation of NR2C(SVA-AIG) was not successfully made. However, the report of Raditsch et al. (1993) suggests that the subunit differences in affinity for the non-competitive, channel-blocking antagonists of the NMDA receptor have a more complicated origin than the amino acid differences within the M2 region. A comparison of the amino sequences of the NMDA receptor subunits shows that the M3 region is also highly conserved, see Figure 1.5. The study by Ferrer-Montiel et al. (1995) demonstrated that the alanine residue at 627 in the NR1 subunit, M3 region was important for high affinity MK801 binding. The alanine identified as important by Ferrer-Montiel et al. (1995) is conserved across all of the NMDA receptor subunits. However, there is a single residue difference between the NR2A and NR2C subunits within the M3 segment, NR2A has a serine at position 610, whilst the equivalent NR2C residue is leucine, see Figure 1.5. Further mutagenesis studies are needed to confirm this.

In conclusion, three individual point mutations of the NR2C subunit were successfully made and expressed in HEK 293 cells. However, they did not significantly alter the phenotype of the NR1a/NR2C cloned receptor with respect to [³H]MK801 binding or cytotoxicity, when expressed in HEK 293 cells.
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General Discussion
The aim of this project was to establish a recombinant model of the native cerebellar NMDA receptor population in mammalian cells. This would allow the identification of the subunits present in the native cerebellar NMDA receptor, although not their stoichiometry. A recombinant model of the native cerebellar NMDA receptor would also allow easier biochemical and pharmacological characterization of the receptor subtype. Manipulation of the cloned receptor by mutagenesis could allow the identification and study of ligand binding sites and subunit-subunit interactions necessary for functional receptors. Following the cloning of the NMDA receptor subunits (Moriyoshi et al., 1991; Meguro et al., 1992; Ikeda et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993) the properties of cloned NMDA receptors were characterized electrophysiologically, primarily in *Xenopus* oocytes. Biochemical analysis of cloned NMDA receptors needed greater levels of expression, therefore a mammalian cell system was utilized.

To allow accurate comparison of the expressed recombinant NMDA receptors with the native NMDA receptors, the initial studies characterized the native mouse cerebellar NMDA receptor (2.3.1). Radioligand binding with $[^3H]MK801$ gave a $K_d$ for the cerebellum which was significantly different from native mouse forebrain. Displacement studies with different compounds further confirmed the existence of distinct NMDA receptor populations in cerebellum and forebrain. This concurred with the report of Ebert et al. (1991).

Previous studies had demonstrated the efficacy of the HEK 293 cell line with a CMV promoter/enhancer vector system for the transient expression of the NMDA receptor subunits (Chazot et al., 1992; Cik et al., 1993). *In situ* hybridization studies had shown the restricted expression of the NR2C subunit to the cerebellum (Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993). Therefore the mouse NR2C cDNA was subcloned into the pCIS mammalian expression vector (2.3.2). The rat NR1a and mouse NR2A cDNAs

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had previously been subcloned into the pCIS vector (Chazot et al., 1992; Cik et al., 1993). The determination of transfection efficiency was made with the control vector pSV-β-galactosidase. The NR2C subunit was successfully expressed in HEK 293 cells as demonstrated by immunoblotting with specific anti-NR2C antibodies (2.3.3). The NR2C polypeptide was N-glycosylated with a molecular mass of 145000 Da. When the expressed NR2C polypeptide was N-deglycosylated a single band of M₆ 133000 was generated. This was in agreement with the predicted molecular weight deduced from the cDNA (Kutsuwada et al., 1992). No specific [³H]MK801 binding was detected in HEK cells singly transfected with the NR2C clone. This concurred with the report of Cik et al. (1991) of a lack of [³H]MK801 binding in HEK 293 cells expressing only the NR2A subunit and the general finding in electrophysiological studies of no functional currents following the expression of only the NR2 subunits in Xenopus oocytes or mammalian cells (e.g. Meguro et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992).

Co-transfection of the NR1a and NR2C clones resulted in co-expression of both subunits, as shown by immunoblotting with subunit-specific antibodies (2.3.4). Immunoprecipitation studies confirmed the two subunits co-assembled in a single oligomer (Chazot et al., 1994). Interestingly, when NR2C was co-transfected with the NR1a clone, immunoblotting detected both N-glycosylated and non-N-glycosylated polypeptides of the NR2C subunit, see Figure 2.3.11. Whereas, expression of NR2C subunit alone allowed detection of only the glycosylated polypeptide, see Figure 2.3.9. The presence of glycosylated and non-glycosylated polypeptide suggests increased expression of the NR2C subunit when NR1a and NR2C cDNAs are co-transfected. Further experiments to confirm if the presence of one subunit influences the level of expression of a second subunit should be performed. Moreover, the determination of where, in the sequence of transcription, translation and assembly of the NMDA receptor subunits, this influence is exerted would be interesting.
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Low levels of specific radioligand binding with $[^3H]MK801$ were detected in cell homogenates prepared from HEK 293 cells co-transfected with pCISNR1a and pCISNR2C. Moreover, the ratio of cDNAs used for transfection was important for obtaining maximal levels of $[^3H]MK801$ binding (2.3.4.1). However, the $K_D$ for $[^3H]MK801$ binding to recombinant NR1a/NR2C receptors was significantly different from that found for native cerebellar NMDA receptors (2.3.4.2). Interestingly the co-expression of NR1a and NR2C subunits did not affect the viability of the HEK 293 cells, unlike previous reports upon the co-expression of NR1a and NR2A polypeptides (Cik et al., 1993). This was presumed to be due to the greater permeant block of the NR1a/NR2C channels by $\text{Ca}^{2+}$, rather than reduced receptor activation by L-glutamate and glycine or increased block by $\text{Mg}^{2+}$ (Burnashev et al., 1995; Ishii et al., 1993; Kutsuwada et al., 1992). Further studies to identify the location of this $\text{Ca}^{2+}$-block site should be performed.

*In situ* hybridization studies reported the presence of NR2A mRNA in the cerebellum as well as NR1 mRNA and NR2C mRNA (Monyer et al. 1994). Therefore, to improve the recombinant model of the native cerebellar NMDA receptor, the NR2A cDNA was included in the HEK 293 cell transfections (2.3.5). Immunoblotting detected the presence of all three subunits following co-transfection. Immunoprecipitation studies on HEK 293 cell homogenates following triple transfections with the NR1a, NR2A and NR2C cDNAs showed the co-association of NR1a with NR2A subunits and the co-association of NR1a and NR2C subunits, moreover, the co-association of the NR2A subunit with the NR2C subunits was also demonstrated (Chazot et al., 1994). These studies together with the radioligand binding data imply the co-assembly of the NR1a, NR2A and NR2C subunits in a single receptor. It was also noted that HEK 293 cells co-transfected with NR1a, NR2A and NR2C required the presence of AP5 post-transfection to prevent cell death. Radioligand binding studies with $[^3H]MK801$ showed the ratio of DNA used for the triple transfections influenced
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the pharmacological properties of the expressed receptor populations (2.3.5.2). These studies also showed that the triple subunit NMDA receptor populations had significant differences in the binding or inhibition constants of the ligands examined as compared to the NR1a/NR2A or NR1a/NR2C cloned receptors. Correlation curves of the rank order of specificity for native cerebellar NMDA receptors and the triple subunit cloned NMDA receptors demonstrated that transfection of HEK 293 cells with NR1a, NR2A and NR2C cDNAs with a ratio of 1:3:3 generated the best model of the native cerebellar NMDA receptors for the ligands so far examined (2.3.5.3). The radioligand binding data and the immunoprecipitation studies suggested that in the native cerebellar NMDA receptor population, the majority of receptors have at least one copy of the NR1, NR2A and NR2C subunits. The molecular size of native NMDA receptors is in the range of 710 - 850 kDa (Brose et al., 1993) and for recombinant NMDA receptors expressed in HEK 293 cells, 780 - 850 kDa (Chazot et al., 1994). The report of Behe et al. (1995) showed that recombinant heteromeric NMDA receptors in *Xenopus* oocytes assemble with two NR1 subunits and a currently undetermined number of NR2 subunits. Beneviste and Mayer (1991) also established that 2 molecules of both L-glutamate and glycine were needed to activate the native NMDA receptor in hippocampal neurons, which together with the evidence of high affinity glycine binding to homomeric NR1 receptors (Grimwood et al., 1995) suggests the presence of two NR1 subunits in the native NMDA receptor. The results in this thesis would be consistent with either three or four NR2 subunits per receptor oligomer. Various combinations of NR2A and NR2C subunits, with the proposed two NR1a subunits, would be consistent with the determined size range of the expressed, recombinant receptor. Moreover, the effect of varying the DNA ratios for the triple transfections altered the pharmacological properties of the expressed receptor population (2.3.5.1). This could result from manipulation of the subunit stoichiometry of the recombinant NMDA receptor or from expression of heterogenous populations of receptors.

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Recombinant NMDA receptors of stoichiometry; \((\text{NR}_{1a})_2(\text{NR}_{2A})_2(\text{NR}_{2C})_1\) through to \((\text{NR}_{1a})_2(\text{NR}_{2A})_1(\text{NR}_{2C})_3\) would be consistent with the determined molecular size. The stoichiometry of the NR2 subunits needs to be established, possibly by using peptide 'tags' and immunoblotting to label subunits in recombinant receptors.

These results demonstrate that distinct types of NMDA receptors are created by the co-expression of different NR2 subunits with the NR1a subunit in HEK 293 cells. Differences were seen in the \(^1H\)MK801 pharmacology and in the cytotoxic effects of the recombinant receptors. Site-directed mutagenesis is one approach to elucidate the reasons for these differences. It has already been demonstrated that the amino acid N598 within the M2 region of the NR1a subunit is responsible for the control of Ca\(^{2+}\) permeability, Mg\(^{2+}\) block and block by MK801 (Sakurada et al., 1993). Previous studies co-expressing the NR1a(N598Q) mutant and the wild-type NR2A subunits showed a significant decrease in cell death as compared to the wild-type NR1a/NR2A receptor (Cik et al., 1994). However, this asparagine is conserved across the three subunits NR1a, NR2A and NR2C and does not therefore explain the differences in MK801 binding affinity and of cell death seen between the NR1a/NR2A and the NR1a/NR2C recombinant receptors.

In this study, mutations were generated within the M2 region of the NR2C subunit and their effects on \(^1H\)MK801 binding and cell death tested. The NR2C subunit was mutated to resemble the NR2A subunit by individual point mutations (3.3.1.3). None of the single amino acid mutations achieved appeared to have any significant effect upon \(^1H\)MK801 binding or cell death following co-transfection with the NR1a cDNA (3.3.2 and 3.3.3). Attempts to replace in NR2C all of the amino acids in the M2 region which differed from NR2A were unsuccessful, as were attempts to replace the negatively charged glutamate (E599) in NR2C with the uncharged glutamine (Q602) in NR2A (3.3.1.3). These mutations need to be achieved before ruling out the M2 region as generating the
subunit-specific differences in $[^3]$HMK801 binding and cytotoxic effects of the NR1a/NR2A and NR1a/NR2C recombinant receptors. However, an investigation by Raditsch et al. (1993) into the subunit-specific differences in ATX binding and blockade of heteromeric NMDA receptors expressed in *Xenopus* oocytes showed neither of the above mutations had a significant effect upon the affinity of the toxin blockade. Furthermore, there is increasing evidence that the highly conserved M3 region of the NMDA receptor subunits is important in the channel properties of the receptor (Ferrer-Montiel et al., 1995). As the detailed crystal structure of the NMDA receptor and its subunits is not yet known, further mutagenesis experiments should also look at other membrane regions and their influence upon the NMDA channel pore.

All of the experiments in this thesis were performed with the NR1a splice variant. Further studies to investigate the role of the other splice variants in the pharmacology of the recombinant NMDA receptors should be performed. There is evidence that the major NR1 splice variant in the cerebellum is NR1g, (Laurie and Seeburg, 1994b). It has also been shown that the NR1a splice variant requires the presence of NR2 subunits for cell surface expression in mammalian cell lines (McIlhinney et al., 1995). It would be of interest to determine if this is the case for all the NR1 splice variants and what mechanisms are controlling the correct assembly and placement of the NMDA receptors. Other studies have shown the importance of the C-terminus in the targeting and aggregation of the NR1 and NR2 subunits to discrete areas of the plasma membrane (Ehlers et al., 1995; Kornau et al., 1995).

The development of model systems for the different native NMDA receptor populations allows the study of the structural elements involved in generating receptor heterogeneity. The model system detailed in this study established the presence of three subunit types within the native cerebellar NMDA receptor. This model may be further refined by looking at the role of the
different NR1 splice variants and their effects on the pharmacology of the recombinant receptor. Furthermore this model system may aid in identifying the elements and mechanisms important in controlling receptor maturation and assembly.
REFERENCES
REFERENCES

Selective modulation of NMDA responses by reduction and oxidation.

Differential expression of five N-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rat.

Combinatorial RNA splicing alters the surface charge on the NMDA receptor.

The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurons by N-methyl-D-aspartate.

The role of divalent cations in the N-methyl-D-aspartate response of mouse central neurones in culture.

Selective depression of excitatory amino acid induced depolarizations by magnesium ions in isolated spinal cord preparations.

Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors.
REFERENCES

Synaptic plasticity: long-term potentiation in the hippocampus.

Identification of a novel N-methyl-D-aspartate receptor population in the rat medial thalamus.

Determination of NMDA NR1 subunit copy number in recombinant NMDA receptors.

NMDA and non-NMDA receptors are co-localized at individual synapses in cultured rat hippocampus.

Changes in voltage dependence of NMDA currents during development.

Topology profile for a glutamate receptor: Three transmembrane domains and a channel-lining reentrant membrane loop.

A kinetic analysis of antagonist action at NMDA receptors: Two binding sites each for glutamate and glycine.

Multiple effects of spermine on N-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurones.

233
REFERENCES

Mutations at two distinct sites within the channel domain M2 alter calcium 
permeability of neuronal α7 nicotinic receptor.

Bettler, B., Boulter, J., Hermans-Borgmeyer, I., O'Shea-Greenfield, A., Deneris, E., 
583-595.
Cloning of a novel glutamate receptor subunit, GluR5 - expression in the nervous 
system during development.

Bettler, B., Egebjerg, J., Sharma, G., Pecht, G., Hermans-Borgmeyer, I., Moll, C., 
Cloning of a putative glutamate receptor - a low affinity kainate-binding subunit.

AMPA and kainate receptors.

Ligand-gated ion channels in the brain: the amino acid receptor superfamily.

Structural requirements for the development of potent N-methyl-D-aspartic acid 
(NMDA) receptor antagonists.

NMDA receptor expression in the mouse cerebellar cortex.
REFERENCES

Biochemical characterization of a non-N-methyl-D-aspartate glutamate receptor in rat brain.

A synaptic model of memory: long-term potentiation in the hippocampus.

Long-lasting potentiation of synaptic transmission in the dentate area of unanaesthetized rabbit following stimulation of the perforant path.

Long-lasting potentiation of synaptic transmission in the dentate area of anaesthetized rabbit following stimulation of the perforant path.

Stable transfection of the NR1 subunit in Chinese hamster ovary cells fails to produce a functional N-methyl-D-aspartate receptor.

Glycine binding sites and NMDA receptors in brain.

Preneolone sulfate potentiation of N-methyl-D-aspartate receptor channels in hippocampal neurones.

Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1.
REFERENCES

Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit.

Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor.

Fractional calcium currents through recombinant GluR channels of the NMDA, AMPA and kainate receptor subtypes.

N-methyl-D-aspartate exposure blocks glutamate toxicity in cultured cerebellar granule cells.

Immunological detection of the NMDAR1 glutamate receptor subunit expressed in human embryonic kidney 293 cells and in rat brain.

Molecular characterization of N-Methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule.
REFERENCES

An investigation into the role of N-glycosylation in the functional expression of recombinant heteromeric NMDA receptors.

Open-channel block of *N*-methyl-D-aspartate (NMDA) responses by memantine: Therapeutic advantage against NMDA receptor-mediated neurotoxicity.

Protein kinase C reduces Mg\(^{2+}\) block of NMDA-receptor channels as a mechanism of modulation.

Electroporation for the efficient transfection of mammalian cells with DNA.


Expression of NMDAR1-1a(N598Q)/NMDAR2A receptors results in decreased cell mortality.

Cell cytotoxicity and changes in intracellular calcium ions mediated by cloned NMDA receptor subtypes.
REFERENCES

Synaptic plasticity in fear conditioning circuits: Induction of LTP in the lateral nucleus of the amygdala by stimulation of the medial geniculat body.

Excitatory amino acids in synaptic transmission in the Schaffer collateral-comissural pathway of the rat hippocampus.

Chemical excitation of spinal neurones.

2-Amino-5-phosphonovalerate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation.

Structural determinants of barium permeation and rectification in non-NMDA glutamate receptor channels.

Arcaine blocks N-methyl-D-aspartate receptor responses by an open channel mechanism: whole-cell and single-channel recording studies in cultured hippocampal neurons.

GYKI 52466, a 2,3-benzodiazepine, is a highly selective, non-competitive antagonist of AMPA/kainate receptor responses.
REFERENCES

Intracellular calcium concentrations during 'chemical hypoxia' and excitotoxic neuronal injury.

Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C.

Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C.

Excitatory amino acid binding sites in the caudate nucleus and frontal cortex of Huntington's disease.

Identification of a novel NMDA receptor in rat cerebellum.

The role of the NR2C subunit in NMDA receptor-gated channels in mouse cerebellar granule cells.

LTP - a structural model to explain the inconsistencies.
Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA.

Intron sequence directs RNA editing of the glutamate receptor subunit GluR2 coding sequence.

Regulated subcellular distribution of the NR1 subunit of the NMDA receptor.

NMDA-receptor channel diversity in the developing cerebellum.

Lipofection: A highly efficient lipid-mediated DNA transfection procedure.

Molecular design of the N-methyl-D-aspartate receptor binding site for phencyclidine and dizolcipine.

Glycine reverses the antagonism of N-methyl-D-aspartate (NMDA) by 1-hydroxy-3-aminopyrrolidine (HA-966) but not by D-2-amino-5-phosphonovalerate (D-AP5) on rat cortical membranes.
REFERENCES

Glutamate: A neurotransmitter in mammalian brain.

Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the N'-methyl-D-aspartate receptor from rat brain.

HA-966 antagonizes N'-methyl-D-aspartate receptors through a selective interaction with the glycine modulatory site.

Novel glutamate receptor antagonists selectively protect against kainic acid neurotoxicity in cultured cerebral cortex neurons.

Excitatory amino acid-mediated cytotoxicity and calcium homeostasis in cultured neurons.

Molecular cloning and developmental analysis of a new glutamate receptor subunit isoform in cerebellum.

Activation of N'-methyl-D-aspartate receptors by L-glutamate in cells dissociated from adult rat hippocampus.
REFERENCES

Site-specific mutagenesis using synthetic oligodeoxyribonucleotide primers: Optimum conditions and maximum oligodeoxyribonucleotide length

SV40 Transformed simian cells support the replication of early SV40 mutants.

High Efficiency Gene Transfer into Mammalian Cells.

Transient Production of Proteins Using an Adenovirus Transformed Cell Line.

Ion-channel assembly.

Recombinant human NMDA homomorphic NR1 receptors expressed in mammalian cells form a high-affinity glycine antagonist binding site.

The characterization of a stably transfected cell line expressing recombinant human NMDAR1a/NMDAR2A N-methyl-D-aspartate receptors.

Safety and tolerability of the glutamate antagonist CGS 19755 in acute stroke patients.

Activation of the multiple-conductance state chloride channels in spinal neurons by glycine and GABA.
REFERENCES

Effects of sodium glutamate on the nervous system.

CLUSTAL: a package for performing multiple sequence alignment on a microcomputer.

Site-directed mutagenesis by overlap extension using the polymerase chain reaction.

Cloning by functional expression of a member of the glutamate receptor family.

Ca\(^{2+}\) permeability of KA-AMPA gated glutamate receptor channels depends on the subunit composition.

Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor.

N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1.

Cloned Glutamate Receptors.
REFERENCES

Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid.

Quinoxalines: Potent competitive non-NMDA glutamate receptor antagonists.


Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK801: Selective binding to open channels.

Receptor-binding studies, a brief outline.

Mutagenesis at a specific position in a DNA sequence.

Genetic assay for small fragments of bacteriophage ΦX174 deoxyribonucleic acid.

Cloning and expression of the epsilon 4 subunit of the NMDA receptor channel.
REFERENCES

Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits.

A novel non-NMDA receptor antagonist shows selective displacement of low-affinity [3H]kainate binding.

Glycine potentiates the NMDA response in cultured mouse brain neurons.

Voltage-dependent block by intracellular Mg$^{2+}$ of N-methyl-D-aspartate-activated channels.

Antagonism of non-NMDA receptors augments the neuroprotective effect of NMDA receptor blockade in cortical cultures subjected to prolonged deprivation of oxygen and glucose.

Crystal structure for the lysine-, arginine-,ornithine-binding protein (LAO) from Salmonella typhimurium at 2.7 Å resolution.

Molecular cloning and chromosomal localization of the key subunit of the human N-methyl-D-aspartate receptor.
REFERENCES

Expression and characterization of the zeta 1 subunit of the N-methyl-D-aspartate (NMDA) receptor channel in a baculovirus system.

Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes.

Regulation of hippocampal NMDA receptors by magnesium and glycine during development.

Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95.

Different base/base mismatches are corrected with different efficiencies by the methyl-directed DNA mismatch-repair system of *E. coli*.

The gapped duplex DNA approach to oligonucleotide-directed mutation construction.

Excitatory amino acid research in Alzheimer's disease: Enhancement and blockade of receptor functions.
REFERENCES

Rapid and efficient site-specific mutagenesis without phenotypic selection.

Mutational analysis of the glycine-binding site of the NMDA receptor: Structural similarity with bacterial amino acid-binding proteins.

Molecular diversity of the NMDA receptor channel.

Glycine-glutamate interactions at the NMDA receptor: Role of cysteine residues.

Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition.

Regional and developmental heterogeneity in splicing of the rat brain NMDAR 1 mRNA.

Cloning, functional coexpression, and pharmacological characterisation of human cDNAs encoding NMDA receptor NR1 and NR2A subunits.

Ifenprodil blocks N-methyl-D-aspartate receptors by a two-component mechanism.
REFERENCES

Apparent desenitization of NMDA responses in *Xenopus* oocytes involves calcium-dependent chloride current.

Functional kainate-selective glutamate receptors in cultured hippocampal neurons.

Whisker-related neuronal patterns fail to develop in the trigeminal brainstem nuclei of NMDAR1 knockout mice.

A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for beta-galactosidase.

Induction of ornithine decarboxylase by N-methyl-D-aspartate receptor activation is unrelated to potentiation of glutamate excitotoxicity by polyamines in cerebellar granule neurons.

Radioligand binding to central phencyclidine recognition sites is dependent on excitatory amino acid receptor agonists.

Ethanol inhibits NMDA-activated ion current in hippocampal neurons.
REFERENCES

Protein measurement with the Folin phenol reagent.

Mechanisms of DNA uptake by mammalian cells: Fate of exogeously added DNA monitored by the use of fluorescent dyes.

High efficiency polyoma DNA transfection of chloroquine treated cells.

Regulation of inducible and tissue-specific gene expression.

N-methyl-D-aspartate receptor-mediated neuroprotection in cerebellar granule cells requires new RNA and protein synthesis.

AMPA glutamate receptor subunits are differentially distributed in rat brain.

Sequence and expression of a metabotropic glutamate receptor.

Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons.
REFERENCES

Roles for mitotic history in the generation and degeneration of hippocampal neuroarchitecture.

A new method for sequencing DNA.

Voltage-dependent block by Mg$^{2+}$ of NMDA responses in spinal cord neurones.

Sites of action on N-methyl-D-aspartate acid receptors studied using fluctuation analysis and a rapid perfusion technique.


Polyamines potentiate responses of N-methyl-D-aspartate receptors expressed in *Xenopus* oocytes.

Cell surface expression of the human N-methyl-D-aspartate receptor subunit 1a requires the co-expression of the NR2A subunit in transfected cells.
REFERENCES

Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs.

Excitatory amino acid neurotoxicity and neurodegenerative disease.

Biochemical and immunocytochemical characterization of antipeptide antibodies to a cloned GluR1 glutamate receptor subunit: Cellular and subcellular distribution in the rat forebrain.

Two classes of NMDA recognition sites: Differential distribution and differential regulation by glycine.

Pharmacologically-distinct NMDA receptor populations of the cerebellum, medial thalamic nuclei, and forebrain.

Identification and characterization of an N-Methyl-D-Aspartate-specific L-[3H]glutamate recognition site in synaptic plasma membranes.

Glutamate operated channels - developmentally early and mature forms arise by alternative splicing.
REFERENCES

Heteromeric NMDA receptors: Molecular and functional distinction of subtypes.

Developmental and regional expression in the rat brain and functional properties of four NMDA receptors.

Identification by mutagenesis of a Mg(2+)-block site of the NMDA receptor channel.

Structure and function of the NMDA receptor channel.

Molecular cloning and characterization of the rat NMDA receptor.

Distribution of NMDA receptor subunits on rat hippocampal, cerebellar and cortical neurons in culture and in brain slices using subunit specific antibodies.

A single site on the ε subunit is responsible for the change in ACh receptor channel conductance during skeletal development.
REFERENCES

Molecular characterization of a novel retinal metabotropic glutamate receptor mGluR6 with a high agonist selectivity for L-2-amino-4-phosphonobutyrate.

Inhibition of restriction endonuclease Nci I cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis.

A family of glutamate receptor genes - evidence for the formation of heteromeric receptors with distinct channel properties.

Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors.

Gene transfer into mouse lymphoma cells by electroporation in high electric fields.

Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus.

A rapid and highly efficient method for preparation of competent *Escherichia coli* cells.
REFERENCES

Magnesium gates glutamate-activated channels in mouse central neurones.

Evidence for heterogenous glycine domains but conserved multiple states of the excitatory amino acid recognition site of the NMDA receptor: regional binding studies with [³H]glycine and [³H]L-glutamate.

Three-dimensional structures of the periplasmic lysine/arginine/ornithine-binding protein with and without a ligand.

Distribution of the messenger RNA for a metabotropic glutamate receptor, mGluR2, in the central nervous system of the rat.

Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction.

Excitotoxic amino acids and neuropsychiatric disorders.
REFERENCES

Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A.

Receptor Measurement.

Phosphonate analogues of carboxylic acids as amino acid antagonists on rat cortical neurones.

Quinolinic acid: regional variations in neuronal sensitivity.

Zinc selectively blocks the action of N-methyl-D-aspartate on cortical neurones.

The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1.

Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody.

The metabotropic glutamate receptors: structure and functions.
REFERENCES

Regional variations in the pharmacology of the NMDA receptor channel blockers: Implications for the therapeutic potential.

Characterization of a metabotropic glutamate receptor: Direct negative coupling to adenylate cyclase and involvement of a persussis toxin-sensitive G-protein.

The atypical M2 segment of the β subunit confers picrotoxin resistance to inhibitory glycine channels.

Agonist response kinetics of N-methyl-D-aspartate receptors in neurons cultured from rat cerebral cortex and cerebellum: Evidence for receptor heterogeneity.

Transient expression shows ligand gating and allosteric potentiation of GABA<sub>A</sub> receptor subunits.

Subunit-specific block of cloned NMDA receptors by argiotoxin636.

Cooperative modulation of [³H]MK801 binding to the N-methyl-D-aspartate receptor-ion complex by L-glutamate, glycine and polyamines.
REFERENCES


$^3$H-labelled MK801 binding to excitatory amino acid complex from rat brain is enhanced by glycine.

Ifenprodil is a novel type of N-methyl-D-aspartate antagonist: Interaction with polyamines.

Multiple sites for the regulation of the N-methyl-D-aspartate receptor.

Regional variations in [$^3$H]MK801 binding to rat brain N-methyl-D-aspartate receptors.

Transmembrane topology of the glutamate receptor subunit GluR6.

The polyamine diaminodecane (DA-10) produces a voltage-dependent flickery block of single NMDA receptor channels.

Spermine and related polyamines produce a voltage-dependent reduction of N-methyl-D-aspartate receptor single-channel conductance.

The polyamine spermine has multiple actions on N-methyl-D-aspartate receptor single-channel currents in cultured cortical neurons.
REFERENCES


Excitotoxicity and the NMDA receptor - still lethal after eight years.


Glutamate receptor RNA editing *in vitro* by enzymatic conversion of adenosine to inosine.


Optimization of the annealing temperature for DNA amplification *in vitro*.


Functional expression from cloned cDNAs of glutamate receptor species responsive to kainate and quisqualate.


Reduced hippocampal LTP and spatial learning in mice lacking NMDA receptor ε1 subunit.


Alteration of Ca²⁺ permeability and sensitivity to Mg²⁺ and channel blockers by a single amino acid substitution in the N-methyl-D-aspartate receptor.


Regionally distinct N-methyl-D-aspartate receptors distinguished by quantitative autoradiography of [3H]MK-801 binding in rat brain.

REFERENCES

DNA sequencing with chain-terminating inhibitors.

D,L-(tetrazol-5-yl) glycine: a novel and highly potent NMDA receptor agonist.

Neuronal-astrocytic interactions in glutamate metabolism.

Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: Role of the glutamate receptor responsive to N-methyl-D-aspartate.

2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline: A neuroprotectant for cerebral ischemia.

Changing subunit composition of heteromeric NMDA receptors during development of rat cortex.

Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: an in situ hybridization study in adult and developing rat.
REFERENCES


A conformationally restricted analogue of L-glutamate, the (2S,3R,4S) isomer of L-α-(carboxycyclopropyl) glycine, activates the NMDA receptor more markedly than NMDA in isolated rat spinal cord.


Glutamate stimulates inositol phosphate formation in striatal neurones.


Glycine potentiates N-methyl-D-aspartate induced [³H]TCP binding to rat cortical membranes.


Flip and flop: A cell-specific functional switch in glutamate-operated channels of the CNS.


RNA editing in brain controls a determinant of ion flow in glutamate-gated channels.


A glutamate receptor channel with high affinity for domoate and kainate.


Polyamines potentiate NMDA induced whole-cell currents in cultured striatal neurons.
REFERENCES

Mechanisms of memory.

Neurol. 343, 1-16.
Organization of N-methyl-D-aspartate glutamate receptor gene expression in the basal
ganglia of the rat.

Single-channel conductances of NMDA receptors expressed from cloned cDNAs:
Comparison with native receptors.

476.3, 391-397.
Single channel properties of cloned NMDA receptors in a human cell line: Comparison
with results from Xenopus oocytes.

Sucher, N.J., Brose, N., Deitcher, D.L., Awobuluyi, M., Gasic, G.P., Bading, H.,
268, 22299-22304.
Expression of endogenous NMDAR1 transcripts without receptor protein suggests
post-transcriptional control in PC12 cells.

Structures and properties of seven isoforms of the NMDA receptor generated by
alternative splicing.

A new type of glutamate receptor linked to inositol phospholipid metabolism.
REFERENCES

Identification of two cysteine residues that are required for redox modulation of the NMDA subtype of glutamate receptor.

Short-term, high efficiency expression of transfected DNA.

Does a radioligand bind to a homogenous population of non-interacting receptor sites?

DNA sequence analysis with a modified bacteriophage T7 DNA polymerase.

Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by *in vitro* mutagenesis.

Signal transduction, pharmacological properties, and expression patterns of two rat metabotropic glutamate receptors, mGluR3 and mGluR4.

Pharmacology of systemically administered domoic acid in mice.

The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA.

262
REFERENCES

The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA.

Glycine is a co-agonist at the NMDA receptor/channel complex.

Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain.

Proton inhibition of N-methyl-D-aspartate receptors in cerebellar neurons.

Relief of experimental spasticity and anxiolytic/anticonvulsant actions of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline.

Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons.

The N-terminal domains of Acetylcholine receptor subunits contain recognition signals for initial steps of receptor assembly.

---

263
REFERENCES

Production of single-stranded plasmid DNA.

[^H]Thienyl-phencyclidine ([^H]TCP) binds to two different sites in rat brain. Localization by autoradiography and biochemical techniques.

Preferential co-assembly of recombinant NMDA receptors composed of three different subunits.

Identification of amino acids in the N-Methyl-D-aspartate Receptor NR1 subunit that contribute to the glycine binding site.

Developmental changes in distribution of NMDA receptor channel subunit mRNAs.

The synthesis of some acidic amino acids possessing neuropharmacological activity.

Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists.
REFERENCES

Immunocytochemical characterization of the non-NMDA glutamate receptor using subunit-specific antibodies - evidence for a heteroligomeric structure in rat brain.

Biochemical and assembly properties of GluR6 and KA2, two members of the kainate receptor family, determined with subunit-specific antibodies.

Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurones.

Effects of polyamine on the binding of [^3H]MK801 to the N-methyl-D-aspartate receptor: Pharmacological evidence for the existence of a polyamines recognition site.

Characterization of polyamines having agonist, antagonist, and inverse agonist effects at the polyamine recognition site of the NMDA receptor.

Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro.

Sensitivity of the N-Methyl-D-Aspartate receptor to polyamines is controlled by NR2 subunits.

265
REFERENCES

Transmembrane topology of two kainate receptor subunits revealed by N-glycosylation.

A unique synaptosomal fraction which accumulates glutamic and aspartic acids in brain tissue.

The anticonvulsant MK801 is a potent N-methyl-D-aspartate antagonist.

Differential modulation by cyclothiazide and concanavalin A of desensitization at native alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and kainate-preferring glutamate receptors.

Different sensitivities of NMDA receptor channel subtypes to non-competitive antagonists.

Cloning, expression and modulation of a mouse NMDA receptor subunit.

Heterogeneity of the N-methyl-D-aspartate receptor ionophore complex in rat brain, as revealed by ligand binding techniques.
REFERENCES

Excitatory amino acid receptors in the brain: membrane binding and receptor autoradiographic approaches.

Spermine potentiation of recombinant N-methyl-D-aspartate receptors is affected by subunit composition.

Mutagenesis rescues spermine and Zn$^{2+}$ potentiation of recombinant NMDA receptors.

Oligonucleotide-directed mutagenesis: A simple method using two oligonucleotide primers and a single-stranded DNA template.
PUBLICATIONS

Refereed Papers;
Molecular Characterization of N-Methyl-D-aspartate Receptors Expressed in Mammalian Cells Yields Evidence for the Coexistence of Three Subunit Types within a Discrete Receptor Molecule
J. Biol. Chem. 269, 24403-24409

Using Promega's CytoTox 96™ Assay System to Measure Cell Death Mediated by NMDA Receptor Subtypes Following Transient Expression in Mammalian Cells
Promega Profiles 20, 8

Abstracts;
Characterization of the Polyamine Modulatory Site on Recombinant NR1/NR2A Receptors

Evidence for the Coexistence of Three Subunit Types in Cerebellar NMDA Receptors
Cambridge Symposia: Molecular Neuroscience

Expression of Heteromeric NMDA Receptor Subtypes in Mammalian Cells Results in Differential Cell Death
Brain Research Association
Molecular Characterization of NMDA Receptors Expressed in Mammalian Cells Yields
Evidence for the Coexistence of Three Subunit Types in Cerebellar NMDA Receptors
American Society for Neuroscience, Neurosci. Abs. 73.3

Chazot, P.L., Cik, M., Coleman, S.K., Langley, D., Pearce, B. and
Cell Cytotoxicity; A Novel Assay System for the Characterization of Functional Cloned
NMDA Receptor Subtypes
American Society of Neurochemistry, J. Neurochem. (suppl.)

Properties of Native and Cloned NMDA Receptor Subtypes: A Biochemical and
Immunological Approach
Molecular Neurobiology - Receptors: Regulation, Function and Signal
Transduction (Italy)

Cik, M., Langley, D., Coleman, S.K., Chazot, P.L., Pearce, B. and
Changes in Intracellular Calcium Mediated by Cloned NMDA Receptor Subtypes
Brain Research Association

Cik, M., Langley, D., Chazot, P.L., Coleman, S.K., Pearce, B. and
Cell Cytotoxicity and Changes in Intracellular Calcium Ions Mediated by Cloned
NMDA Receptor Subtypes
An Investigation into the Ratios of NR1, NR2A and NR2C clones used for Mammalian Cell Transfection on the Pharmacological Profile of the Resultant Recombinant NMDA Receptor Population
American Society for Neuroscience, Neurosci. Abs. 41.6
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Cambridge, CB2 2Q8 by 15 July, 1994.)
EVIDENCE FOR THE COEXISTENCE OF THREE SUBUNIT TYPES IN CEREBELLAR NMDA RECEPTORS

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The N-methyl-D-aspartate (NMDA) receptors are a subclass of ionotropic, excitatory glutamate receptors. They have been implicated in synaptic plasticity, probably involving some types of learning and memory formation, epilepsy and neurodegeneration. Recent cloning has shown the existence of five NMDA receptor genes, NMDA R1 and NMDA R2A-D (Hollmann and Heinemann, 1994). Combinations of these are thought to coassemble to form functional receptors. As an approach to determining which subunits coexist in the brain, we have expressed different subunit combinations transiently in mammalian cells and compared their radioligand binding properties to those of adult mammalian brain. In this report, we focus on the cerebellar receptors where in situ hybridisation studies have found high levels of expression of NMDA R1 and NMDA R2C mRNAs. [3H]MK801 binding to adult mouse cerebellum has a $K_0 = 22 \pm 9$ nM. However, human embryonic kidney (HEK) 293 cells cotransfected with NMDA R1 and R2C clones yielded a $K_0 = 345 \pm 158$ nM. Triple transfection with NMDA R1, R2C and R2A clones at a defined DNA ratio yielded a $K_0 = 22 \pm 5$ nM. This value was in agreement with that of native cerebellar receptors and significantly different to the $K_0$ of [3H]MK801 binding to mouse forebrain, i.e. $K_0 = 5 \pm 0.2$ nM. Further characterisation of the pharmacological specificity of the triple transfected HEK 293 cells showed a correlation coefficient $r = 0.992$ between the $K_i$ values of adult cerebellum and the transfected cells for a series of six disparate compounds active at NMDA receptors. The coassociation of these three subunits in the transfected cells was demonstrated by immunoprecipitation studies using subunit-specific antibodies (Chazot et al., 1994). These results substantiate the report of Wafford et al. (1993) who suggested the preferential assembly of these three subunit types in Xenopus oocytes. Furthermore, the coexistence of three NMDA receptor subunit types agrees with the findings of Sheng et al. (1994) for NMDA receptors of adult rat forebrain.

SC holds an SERC postgraduate studentship.

AN INVESTIGATION INTO THE RATIOS OF NR1, NR2A AND NR2C CLONES USED FOR MAMMALIAN CELL TRANFECTION ON THE PHARMACOLOGICAL PROFILE OF THE RESULTANT RECOMBINANT NMDA RECEPTOR POPULATION. S. Coleman, P.L. Chazot, M. Cik and F.A. Stephenson.

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We have previously reported pharmacological and immunological evidence for the coassociation of three NMDA receptor subunit types within a single oligomer following the transient coexpression of the NR1, NR2A and NR2C polypeptides in human embryonic kidney (HEK) 293 cells (1). During that study, it was found that the ratios of the respective clones used for mammalian cell transfection influenced the pharmacological properties of the expressed receptor. In double combination transfections, NR1/NR2A had a $K_i = 7 \pm 2$ nM for $[^3H] MK801$ binding compared to $K_i = 34 \pm 158$ nM for NR1/NR2C receptors. In triple combinations a 1:3:10 NR1/NR2A/NR2C DNA ratio yielded a receptor which was reminiscent of NR1/NR2C with respect to $[^3H] MK801$ binding. In contrast, 1:3:3 and 1:10:3 NR1/NR2A/NR2C transfections yielded NMDA receptors which were not distinguished on the basis of their $K_i$ for $[^3H] MK801$. To investigate this further, a more detailed pharmacological profile of HEK 293 cells triply transfected with NR1/NR2A/NR2C clones with DNA ratios 1:10:3, 1:3:3 and 1:3:10 was carried out. This included the determination of the $IC_{50}$s and respective $K_i$s for the displacement of $[^3H] MK801$ binding by MK801, ketamine, TCP, Mg$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$. The $K_i$s for this series of compounds were differentially influenced by the DNA ratios used and for each, no evidence for binding site heterogeneity was found. These results may be extrapolated to yield insights into subunit stoichiometry and assembly.

Molecular Characterization of N-Methyl-D-aspartate Receptors Expressed in Mammalian Cells Yields Evidence for the Coexistence of Three Subunit Types within a Discrete Receptor Molecule

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The N-methyl-D-aspartate (NMDA) pharmacological sub-class of glutamate receptor is a fast-acting ligand-gated cation channel with a high permeability for Ca2+. Several genes encoding NMDA receptor subunits have been identified (1–5). In these genes fall into two classes which are defined on the basis of the amino acid sequence homology of the deduced primary structures of the proteins they encode. These are the NMDA R1 and the NMDA R2 receptor subunits. Diversity of the NMDA R1 subunit is created by extensive alternative splicing of the NMDA R1 subunit gene to yield eight isoforms (6), whereas heterogeneity of the NMDA R2 subunit is from the existence of multiple, related genes, NMDA R2A-R2D, (e.g. Refs. 2–5). Expression studies, predominantly utilizing the Xenopus oocyte translation system, have shown that although NMDA R1 can form functional homomeric glutamate-gated channels (e.g. Ref. 1), greatly enhanced glutamate-gated conductances are obtained by the co-expression of the NMDA R1 and an NMDA R2 subunit (2–5). In situ hybridization with subunit-specific probes corroborates the functional expression studies showing that the NMDA R1 mRNA is ubiquitously expressed whereas the NMDA R2 mRNA distributions show distinct but overlapping patterns (3–5). Thus, it has been suggested that NMDA receptors may comprise an NMDA R1 subunit assembled with a subunit of one type of the NMDA R2 class, e.g. an NMDA R1/R2A receptor where the subunit ratios are not known. However, two recent reports have shown evidence for the co-assembly of three NMDA receptor subunit types in one protein. Wafford et al. (7) showed the preferential assembly of the NMDA R1, R2A, and R2C subunits following co-expression in Xenopus oocytes. The resulting recombinant protein differed in glycine sensitivity from both NMDA R1/R2A and NMDA R1/R2C receptors (7). Moreover, Sheng et al. (8) reported results which were consistent with the partial coexistence of NMDA R1, 2A, and 2B subunits in native receptors of adult rat forebrain.

We have established conditions for the optimal transient expression of cloned NMDA R1/R2A receptors in human embryonic kidney (HEK) 293 cells with minimal cell death, thus permitting their detailed biochemical and pharmacological characterization (9). It was of interest to compare the biochemical properties of other cloned NMDA receptor subtypes with those of the wild-type. We focused firstly on cerebellar NMDA receptors because of the restricted, high levels of expression of the NMDA R2C mRNA in cerebellum (3–5). In initial studies, we observed that co-expression of the NMDA R1 and NMDA R2 subunits did not yield a receptor with properties characteristic of adult cerebellar NMDA receptors. Further investigation provided both biochemical and pharmacological evidence for the co-association of the NMDA R1, NMDA R2A, and NMDA R2C subunits whose properties were reflective of native cerebellar NMDA receptors. We report these results in this paper.

EXPERIMENTAL PROCEDURES

Materials—[3H](+)-5-Methyl-10,11-dihydro-dibenzo[a,d]cyclohepten-5,10-imine (MK801; 28.8 Ci/mmol) was from Du Pont (UK) Ltd. (Stevenage, Herts., UK). The peptides Cys NMDAR 2C-(20-34), NMDAR 2C-(1227-1237), and the sequence LQLCSRHRES, was from Multiple Peptide Systems (San Diego, CA). The peptides Cys NMDAR 2C-(20-34), NMDAR 2C-(1227-1237), and the Multiple Antigen Peptide (MAP), NMDAR 2A-(1435-1445AA), MAP were from Peptide and Protein Research (University of Exeter, Devon, UK). Rabbit immunoglobulin, horseradish-linked whole antibody, and the enhanced chemiluminescence (ECL) detection system were from Amersham International (Bucks, UK). N-Glycosidase F was from Boehringer Mannheim (Lewes, East Sussex,
Expression of NMDA R1/NMDA R2C Heteromeric Receptors in HEK 293 Cells —We have previously reported that the maximum expression of NMDA R1/NMDA R2A heteromeric receptors in HEK 293 cells, determined by both [3H]MK801 binding activity and immunoblotting with subunit-specific antibodies, was dependent upon the ratio of the respective DNAs used and the presence of AP5 in the cell culture media post-transfection (9). Thus, in initial transfection studies for the expression of NMDA R1/NMDA R2C heteromeric receptors, the respective plasmid ratios were varied and the cells were screened for binding activity and immunoreactivity. The DNA ratios employed were NMDA R1:NMDA R2C, 4:1, 1:1, 1:4, 1:7, 1:10, and 1:14. Single-step plasmid transfections were performed. The transfection assays were carried out in 3-15% polyacrylamide slab gels following the method of Ref. 13. The ratio of NMDA R1:NMDA R2C in the transfected cells was determined by double immunoblotting with subunit-specific antibodies. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone. N-Deglycosylation of Recombinant NMDA R2C—Homogenates of HEK 293 cells transfected with plasmid pCR3NMDAR2C (60 pg of protein) were incubated in the presence and absence of N-Glycanase (final concentration 20 units/ml) for 16 h at 4 °C with 100 µg/ml respective peptide in phosphate-buffered saline or with phosphate-buffered saline alone.
4 is cell homogenates incubated at 37 °C for 4 h prior to analysis, and blotting are: NMDA Rl, R2A, and R2C clones and the antibodies used for immunoblotting with anti-NMDA Rl (929-938); anti-NMDA R2A (1435-1445); 3 and 4, anti-NMDA R2C (1227-1237); 5 and 6, anti-Cys NMDA R2C (20-34); 7 and 8, anti-NMDA R2C (1227-1237). Lane 7 is cell homogenates incubated at 37 °C for 4 h prior to analysis, and lane 8 is cell homogenates treated with K⁺ glycose for 4 h at 37 °C. In lanes 4 and 6, antibody was preincubated overnight at 4 °C with the respective peptide that was used for antibody production. The positions of molecular weight markers (x 10³) are shown on the left.

![Figure 1](image_url)

**Figure 1.** Characterization of the anti-NMDA R2C subunit-specific antibodies by immunoblotting. Cell homogenates were prepared from HEK 293 cells transfected with the calcium phosphate method with pcISNMDA R1, pcISNMDA R2A, and NMDA R2C with a total of 20 μg of DNA. Immunoblotting was carried out in 6% SDS-PAGE under reducing conditions with 60 μg of protein applied per gel lane, transfer to nitrocellulose, and using the ECL detection system all as described under “Experimental Procedures.” Affinity-purified antibodies were employed at final concentrations of 2-10 μg/mL. Lanes containing 60 μg of protein from HEK 293 cell homogenates co-transfected with the NMDA R1, R2A, and R2C clones and the antibodies used for immunoblotting are: 1, blotting with anti-NMDA R1 (929-938); 2, anti-NMDA R2A (1435-1445); 3 and 4, anti-NMDA R2C (1227-1237); 5 and 6, anti-Cys NMDA R2C (20-34); 7 and 8, anti-NMDA R2C (1227-1237). Lane 7 is cell homogenates incubated at 37 °C for 4 h prior to analysis, and lane 8 is cell homogenates treated with K⁺ glycose for 4 h at 37 °C. In lanes 4 and 6, antibody was preincubated overnight at 4 °C with the respective peptide that was used for antibody production. The positions of molecular weight markers (x 10³) are shown on the left.

**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kᵢ</th>
<th>Bₘ₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse forebrain</td>
<td>5.4 ± 0.2</td>
<td>1809 ± 157</td>
</tr>
<tr>
<td>Mouse cerebellum</td>
<td>22.0 ± 0.7</td>
<td>552 ± 215</td>
</tr>
<tr>
<td>NMDA R1</td>
<td>8.8 ± 2.5</td>
<td>113 ± 37</td>
</tr>
<tr>
<td>NMDA R1/2A</td>
<td>7.0 ± 2.4</td>
<td>976 ± 299</td>
</tr>
<tr>
<td>(1:3) + AP5 (407-1477)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDA R1/2C</td>
<td>346.0 ± 158</td>
<td>1065 ± 405</td>
</tr>
<tr>
<td>(1:10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDA R1/2A/2C</td>
<td>12.0 ± 6.0</td>
<td>962 ± 77</td>
</tr>
<tr>
<td>(1:10):+AP5</td>
<td>(913-1051)</td>
<td></td>
</tr>
<tr>
<td>NMDA R1/2A/2C</td>
<td>22.0 ± 5.0</td>
<td>486 ± 190</td>
</tr>
<tr>
<td>(1:3) + AP5 (243-816)</td>
<td></td>
<td></td>
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</tbody>
</table>

* Results from Ref. 10.
* Results from Ref. 15.
* The degree of significance between the Kᵢ values was p < 0.01.
* Values in brackets show the range of Bₘ₀ values.
* The Kᵢ was determined by displacement with unlabeled MK801 using [³H]MK801 at a final concentration of 50 nM.
* NS is nonsaturating and ND is not determined.

Results from Ref. 10. Results from Ref. 15. The degree of significance between the Kᵢ values was p < 0.01. Values in brackets show the range of Bₘ₀ values. The Kᵢ was determined by displacement with unlabeled MK801 using [³H]MK801 at a final concentration of 50 nM. NS is nonsaturating and ND is not determined.

**293 Cells**—Since both the dissociation constant for the binding of [³H]MK801 to NMDA R1/NMDA R2C cloned receptors was significantly different from the value found for adult cerebellum (Table I) and a recent report had suggested the preferential co-assembly of the NMDA R1/NMDA R2A and the NMDA R2C subunits following expression in Xenopus oocytes (7), triple transfections of HEK 293 cells were carried out. The ratios of DNAs used for HEK 293 cell triple transfections were varied, and the dissociation constants for the binding of [³H]MK801 to these various expressed receptors were determined. In all cases, the results were best fit by the binding of [³H]MK801 to a single site. The Kᵢ values are summarized in Table I together with values for the native receptor. For all transfection experiments, the expression of the respective subunits was verified by immunoblotting with subunit-specific antibodies (results not shown). From Table I, it can be seen that in agreement with previous work (19), the Kᵢ for the binding of MK801 to the cerebellar NMDA receptor is 4-fold lower than to the receptor of mouse forebrain. The Kᵢ for the binding of [³H]MK801 to the NMDA R1/2A/2C cloned receptor for DNA transfection ratios of 1:3:3 agrees with that for the native cerebellar NMDA receptor (Table I). A DNA ratio of 1:3:10 results in a low affinity receptor similar to that found for NMDA R1/2C cloned receptors.

In the comparison between the pharmacological specificity of the forebrain and the NMDA R1/R2A receptor, there is good agreement between the respective inhibitory constants for six drugs tested with the exception of Mg²⁺ which showed an approximate 14-fold lower affinity for the recombinant receptor. The correlation coefficients for the comparison of the rank order of potency between the forebrain receptor and NMDA R1/R2A was r = 0.98 including Mg²⁺ and r = 0.995 excluding the value for Mg²⁺. For the comparison between the cerebellar receptor and NMDA R1/R2A/R2C, for six compounds tested, the correlation coefficient was r = 0.992 (Fig. 2).

The **Percentage Cell Death following NMDA Receptor Subtype Expression in HEK 293 Cells**—It was earlier observed that the expression of the NMDA R1/R2A receptor in HEK 293 cells resulted in cell death which was prevented by the inclusion of 200 μM AP5 in the cell culture media post-transfection (9). In contrast, we observed that the co-expression of the NMDA R1 and NMDA R2C receptor subunits in the same cell line apparently did not result in significant cell death. The percentage cell death was thus quantified for the NMDA R1/R2C combination and for the triply transfected cells. The results are summarized in Table III. The percentage cell death of all combinations was calculated with respect to the expression of the NMDA R1/R2A subtype which we have previously reported resulted in 100% death of transfected cells (15). From Table III, it can be seen that indeed there is no detectable cell death when the NMDA R1 and R2C subunits are co-expressed; thus, all subsequent experiments using this double combination did not require the presence of 200 μM AP5 post-transfection. In contrast, for the co-expression of the NMDA R1/2A/2C subunits in DNA ratios of 1:3:3 and 1:10:3, there is no significant difference in percentage cell death compared to the 100% for the NMDA R1/R2A combination. For the DNA ratio 1:3:10 that yields a recombinant receptor with a low affinity for [³H]MK801, the percentage cell death is reduced by approximately 50% (Table III). Note that for all combinations studied, the number of receptors expressed per mg of protein were in a similar range (Table I).
Assemble of Three Subunit Types in Cerebellar NMDA Receptors

The inhibitory constants were determined all as described under "Experimental Procedures." The concentration of [3H]MK801 used for the determination of the respective inhibitory constants was equal to $K_i$, i.e. either 7 nM or 20 nM. The values are the means ± S.D. for at least n = 3 determinations.

Table II

Comparison of the pharmacological specificity of [3H]MK801 binding to NMDA R1/2A/2C recombinant and native NMDA receptors

<table>
<thead>
<tr>
<th></th>
<th>Inhibitory constants</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MK801</td>
<td>Tenovyclidine</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Mouse forebrain</td>
<td>5.4 ± 0.2</td>
<td>75 ± 30</td>
</tr>
<tr>
<td>NMDA R1/NMDA R2A</td>
<td>7.0 ± 2.4</td>
<td>64 ± 32</td>
</tr>
<tr>
<td>Mouse cerebellum</td>
<td>22.3 ± 9.0</td>
<td>1603 ± 144</td>
</tr>
<tr>
<td>NMDA R1/NMDA R2A/NMDA R2C</td>
<td>22.2 ± 4.9</td>
<td>1609 ± 306</td>
</tr>
</tbody>
</table>

* Dissociation constant.

**The degree of significance between the inhibitory constants was p < 0.01.

DNA ratios used for transfection were 1:3:3 for NMDA R1/2A/2C, respectively.

Fig. 2. Correlation of the rank order of pharmacological specificity between recombinant and native adult NMDA receptors. A shows the correlation of the affinity constants for the inhibition of [3H]MK801 binding for a series of compounds to native NMDA receptors of adult mouse forebrain and NMDA R1/R2A cloned receptors. B shows the correlation of the affinity constants for the inhibition of [3H]MK801 binding for a series of compounds to native NMDA receptors of adult mouse cerebellum and NMDA R1/2A/2C 1:3:3 cloned receptors.

Table III

The percentage cell death following the transient expression of NMDA receptor subtypes in HEK 293 cells

HEK 293 cells were transfected with plasmids containing the respective NMDA receptor subunit cDNAs by the calcium phosphate method. Then 24 h post-transfection, the cell culture supernatant (50 µl) from a fixed number of cells, i.e. 4 x 10⁵ seeded 24 h prior to transfection, was collected and assayed for lactate dehydrogenase activity as all as described under "Experimental Procedures." Values were normalized to take account of the efficiency of transfection. They are expressed as the percentage cell death which was calculated by the determination of the percentage lactate dehydrogenase activity in the different cell culture supernatants with respect to the maximum lactate dehydrogenase activity released from 4 x 10⁵ cells seeded as above. Triplicate values were determined per transfection and the results above show the means ± S.D. for n = 3 separate transfections.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Percentage cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA R1/2A (1:3)</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>NMDA R1/2C (1:10)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:10:3)</td>
<td>106 ± 10</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:3:3)</td>
<td>89 ± 17</td>
</tr>
<tr>
<td>NMDA R1/2A/2C (1:3:10)</td>
<td>55 ± 17</td>
</tr>
</tbody>
</table>

acrylamide gel electrophoresis and immunoblotting using the subunit-specific antibodies. The results are shown in Fig. 3 for the NMDA R1/2A, NMDA R1/2A/2C, 1:3:3 and 1:3:10 receptors. For each combination, immunoblotting with the relevant antibodies showed the presence of a single, diffuse immunoreactive species with a molecular size in the range 780,000–850,000 daltons that was not present in the nontransfected 293 cells.

Demonstration of the Coexistence of the NMDA R1, NMDA R2A, and NMDA R2C Subunits by Immunoprecipitation—A further series of experiments was carried out which were designed to substantiate the results from both the native PAGE and radioligand binding studies of the double and triple transfections. In the first study, HEK 293 cells were transfected with either the NMDA R1/2A or the NMDA R1/2C clones. Cell homogenates were prepared, and immunoprecipitation studies were carried out. The results are shown in Fig. 4. It can be seen that for the NMDA R1/2A transfected cells, immunoprecipitation with the anti-NMDA R1 antibody resulted in the detection of the NMDA R2A subunit in the pellet. Similarly, for NMDA R1/2C transfected cells, following immunoprecipitation with either anti-NMDA R1 or anti-NMDA R2C antibodies, NMDA R2C subunit was detected in the immune pellet. (Immunoprecipitation experiments using the anti-NMDA R2A antibody were not carried out because of antibody availability.) Control experiments using immunoprecipitation with nonimmune Ig did not detect NMDA receptor subunits in the pellets. These results therefore confirm the findings of the PAGE experiments that the two subunit types co-assemble. (Note that the strong immunoreactive bands with $M_r = 50,000$ found with all samples is the immunoglobulin from the precipitation with the primary antibody. Further it is difficult to quantify the signals obtained in the immunoblots because of the differences in the antibody affinities; particularly the anti-NMDA R1 antibody has potent avidity).

HEK 293 cells were then transfected with all three clones, soluble extracts were prepared, and immunoprecipitation assays were carried out with a subunit-specific antibody. The immune pellets were subsequently assayed for the presence of the other two NMDA receptor subunits by immunoblotting using now a different specificity antibody. The results are shown in Fig. 5. It can be seen that following immunoprecipitation with anti-NMDA R1 antibodies, NMDA R1, NMDA R2A, and NMDA R2C subunits were all detected in the immune precipitates. Importantly, immunoprecipitation with anti-NMDA R2C antibodies also showed the presence of the NMDA R1, NMDA R2A, and NMDA R2C polypeptides in the pellets. For both sets of experiments, no NMDA receptor subunits were detected following immunoprecipitation with nonimmune Ig (Fig. 5).

Stringent conditions were required to solubilize the cloned...
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Fig. 3. Molecular size determination of NMDA receptors expressed in HEK 293 cells by native PAGE followed by immunoblotting. HEK 293 cells were transfected with either pCISNMDA R1/R2A or pCISNMDA R1/R2A/R2C with a DNA ratio 1:3:3 and 1:3:10. Cell homogenates were collected 24 or 40 h post-transfection, and native PAGE followed by immunoblotting was carried out as described under “Experimental Procedures.” For each sample, 60 μg of protein was applied per gel lane. Lanes are: 1, 4, and 7, nontransfected cells; 2, 3, 5, and 6, HEK 293 cells transfected with NMDA R1/2A; 8 and 10, HEK 293 cells transfected with NMDA R1/R2A/R2C with DNA ratio 1:3:3; and 9, HEK 293 cells transfected with NMDA R1/2A/2C with DNA ratio 1:3:10. R1 (1, 2, and 5), R2A (3, 4, and 6), and R2C (7–10) are the specificity antibodies used for immunoblotting. For lanes 5, 6, and 10, antibody was preincubated overnight at 4 °C with the peptide used for initial antibody production. The positions of protein standards (×10^6) are shown on the left. The filled arrowhead denotes the immunoreactive band detected by each specificity antibody.

Fig. 4. Immunoprecipitation of NMDA receptors from HEK 293 cells transfected with pCISNMDA R1/pCISNMDA R2A and pCISNMDA R1/pCISNMDA R2C double combinations. HEK 293 cells were co-transfected with either pCISNMDA R1/2A or pCISNMDA R1/R2C. Cells were collected 24 or 40 h post-transfection for NMDA R1/R2A and NMDA R1/R2C, respectively, and solubilized with extraction buffer as described under “Experimental Procedures.” Immunoprecipitation assays were carried out, and the pellets were subjected to immunoblotting with different specificity anti-NMDA receptor antibodies. A shows the results for the pCISNMDA R1/R2A-transfected cells where the lanes are: 1 and 4, HEK 293 cell-solubilized extracts; 2 and 5, pellets obtained with anti-NMDA R1 (929–938) immunoprecipitation; 3 and 6, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1 is blotting with anti-NMDA R1 (929–938) antibodies, and R2A is blotting with anti-NMDA R2A (1435–1445) antibodies. B shows the results for the pCISNMDA R1/R2C-transfected cells where the lanes are: 1 and 5, HEK 293 cell-solubilized extracts; 2 and 6, pellets obtained with anti-NMDA R1 (929–938) immunoprecipitation; 3 and 7, pellets obtained with anti-NMDA R2C (1227–1237) immunoprecipitation; and 4 and 8, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1 is blotting with anti-NMDA R1 (929–938) antibodies, R2A is blotting with anti-NMDA R2A (1435–1445), and R2C is blotting with anti-NMDA R2C (1227–1237) antibodies. For both, the positions of prestained protein standards (×10^6) are shown on the left.

NMDA receptors from the HEK 293 cell membranes with sufficient yield for these experiments. Therefore, control experiments were carried out to show that the co-precipitation of all three NMDA receptor subunits was not an artifact of the extraction procedure. The strategy here followed that used by Wenthold et al. (17). HEK 293 cells were transfected with either the NMDA R1, the NMDA R2A, or the NMDA R2C clone alone. Following harvesting, the three cell populations were pooled, and receptor extraction and immunoprecipitation were carried out as above. The results are shown in Fig. 6. Now, immunoprecipitation with an anti-NMDA R1 antibody did not result in the co-precipitation of either the NMDA R2A or the NMDA R2C polypeptides (Fig. 6). Conversely, immunoprecipitation with the anti-NMDA R2C antibody did not pellet either the NMDA R1 or the NMDA R2A subunits (results not shown).

DISCUSSION

Recently, insights into the functional properties of fast-acting neurotransmitter receptors have been made possible by a comparison between the respective recombinant proteins and those found in situ in the brain. The study of cloned NMDA receptors has to date focused largely on the use of the Xenopus oocyte expression system followed by electrophysiological characterization. Several groups have shown that the co-expression of
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Fig. 5. Immunoprecipitation of NMDA receptors from HEK 293 cells co-transfected with pCISNMDA R1/pCISNMDA R2A/pCISNMDA R2C. HEK 293 cells were co-transfected with pCISNMDA R1/R2A and R2C. Cell homogenates were collected 40 h post-transfection, and solubilized extracts were prepared as described under "Experimental Procedures." Immunoprecipitation assays were carried out, and the resultant immune pellets were subjected to analysis by immunoblotting. Lanes are: 1, 4, and 7, pellets obtained with anti-NMDA R1 (929-938) immunoprecipitation; 2, 5, and 8, pellets obtained with anti-NMDA R2C (1227-1237) immunoprecipitation; 3, 6, and 9, pellets obtained with nonimmune protein A-purified Ig immunoprecipitation. R1, NMDA R2A (1435-1445), and anti-NMDA R2C (1227-1237) antibodies, respectively. The positions of protein standards (x 10^6) are shown on the left.

Fig. 6. Demonstration that the co-immunoprecipitation of three subunits in triple-transfected HEK 293 cells is not an artifact of receptor aggregation. HEK 293 cells were transfected with either pCISNMDA R1, pCISNMDA R2A, or pCISNMDA R2C alone or as a triple combination. Cell homogenates were collected 24 h post-transfection; the singly transfected cells were combined with a cell protein ratio of NMDA R1/R2A/R2C 1:3:3. Solubilized extracts were prepared, immunoprecipitation assays were carried out, and the immune pellets were analyzed by immunoblotting. Lanes are: 1, 3, and 5, pellets obtained from triple subunit combinations after immunoprecipitation with anti-NMDA R1 (929-938) antibodies; 2, 4, and 6, pellets obtained from single-subunit transfections after precipitation with anti-NMDA R1 (929-938) antibodies. R1, R2A, and R2C are immunoblotting with anti-NMDA R1 (929-938), anti-NMDA R2A (1435-1445), and anti-NMDA R2C (1227-1237) antibodies, respectively. The positions of protein standards (x 10^6) are shown on the left.

NMDA R1 with an NMDA R2 subunit is required for robust channel activity, thus suggesting that native receptors are, in the main, heteromeric (e.g. Refs. 2-5). Indeed, we have previously shown a 10-20-fold increase in [3H]MK801 binding sites for NMDA R1/R2A cloned receptors compared to NMDA R1 expressed alone (9). Further, the NMDA R1/NMDA R2 variant receptors have subtly different properties which include sensitivity to both glutamate and glycine, MK801 antagonism, voltage-dependent Mg^2+ block, and polyamine modulation (summarized in Ref. 20). Analysis at the single-channel level of NMDA R1/R2A and NMDA R1/R2C revealed channels with distinct conductance and kinetic properties (21, 22). For both these putative subtypes, a qualitative resemblance to native NMDA receptors was found. In the results reported herein, we have extended our characterization to additional putative cloned NMDA receptor subtypes again expressed transiently in mammalian cells. This included, importantly for a multisubunit protein, an investigation of the HEK 293 cell transfection conditions as well as both biochemical and immunological characterization of the respective expressed and native receptors.

For the NMDA R1 and NMDA R2A double transfection experiments, we showed that the two subunits co-assemble to form a heteromeric receptor with a molecular size of 780,000-850,000 daltons. This is within the range determined by crosslinking and gel filtration studies for native membrane-bound and solubilized NMDA receptors of rat brain by Brose et al. (23). The pharmacological specificity of the cloned NMDA R1/R2A receptor was similar to that found in adult mouse and rat brain. A notable difference was a 14-fold lower affinity for Mg^2+ inhibition of [3H]MK801 binding to the cloned receptor. Lynch et al. (24) recently reported a more detailed pharmacological profile of NMDA R1/NMDA R2A receptors expressed in HEK 293 cells. In their study, differences found between native and cloned receptors included a lack of spermidine stimulation of [3H]MK801 binding and a reduced enhancement of [3H]MK801 binding by Mg^2+. We have made similar observations for the absence of the stimulatory spermidine modulatory site.

In initial experiments with the NMDA R1/R2C combination, respective DNA ratios were varied to optimize maximal transient expression which was assayed for by both [3H]MK801 binding activity and immunoblotting. Under all DNA ratios tested, NMDA R1 and R2C subunits were detected with subunit-specific antibodies. The molecular weight of the NMDA R2C subunit agreed with that predicted from the cDNA sequence and that in brain and after expression in S9 insect cells (27). Furthermore, the molecular size of the cloned protein determined by native gel electrophoresis (results not shown) was in the range found for native receptors. Immunoprecipitation studies further substantiated the co-association of both the NMDA R1 and NMDA R2C subunits in the transfected cells (Fig. 4). However, the affinity of the recombinant receptor for [3H]MK801 was >10-fold lower than found for native receptors (Table I). This reduced affinity is in agreement with electrophysiological studies where a decrease in MK801 sensitivity between NMDA R1/R2A and NMDA R1/R2C receptors was found (28). Triple subunit HEK 293 cell transfections, now including the NMDA R2A subunit at a defined DNA ratio, yielded a receptor with a single high affinity site for [3H]MK801. The determined K_p value was significantly different from that found for forebrain NMDA receptors but correlated with the K_p for cerebellar NMDA receptors (Tables I and II and Fig. 2). Immunoprecipitation assays and native PAGE further substantiated the co-assembly of all three subunit types (Figs. 3-6). It is noted, however, that each of these experimental paradigms does not show definitively the coexistence of the three subunits in one receptor. Double immunoprecipitations or the serial use of different specificity immunospecific antibodies will be required for this (see below). But overall, the radioligand binding results, immunoprecipita-

tion studies, and native PAGE are most consistent with the co-assembly of the NMDA R1, R2A, and R2C subunits to form receptors with properties resembling those of the NMDA receptors of adult cerebellum.

An interesting observation was the fact that despite a similar range of [3H]MK801 binding sites being expressed in the NMDA R1/R2C double transfection experiments, cell death was not found. This may be explained by a decreased sensitivity to l-glutamate activation and/or a decrease in the Ca\(^{2+}\) permeability of this expressed receptor subtype compared to NMDA R1/2A combinations. However, electrophysiological studies have shown that, in fact, NMDA R1/R2C receptors have increased glutamate sensitivity compared to NMDA R1/2A (e.g. Ref. 4) and the two receptors reportedly have similar Ca\(^{2+}\) permeabilities (e.g. Ref. 29). Single-channel studies showed that the NMDA R1/R2C receptors had lower conductance states which may partly explain the differences in cell death (21). Channels with conductance states similar to NMDA R1/R2C were described in mature postmigratory cerebellar granule cells (30). There have been no reports so far of the single-channel properties of expressed NMDA receptors containing three subunit types. Our results here would suggest that, in fact, the majority of cerebellar NMDA receptors comprise at least one copy of three distinct subunits. We cannot, however, exclude the possibility of the existence in the cerebellum of minor NMDA receptor subpopulations containing only two polypeptide types. Such receptors would not be detectable by the methods used herein. Further detailed studies using native detergent-solubilized cerebellar membranes in immunoprecipitation assays and/or the use of different specificity immunofluorescence columns in series are required. Such approaches have been employed successfully for the delineation of the subunit complements of the major inhibitory ligand-gated ion channels, the GABA\(_A\) receptors (e.g. Ref. 13).

In summary, we have described the characterization of NMDA receptor subtypes expressed in mammalian cells. In particular, we have provided biochemical evidence for the co-assembly of three different NMDA receptor subunit types, NMDA R1, NMDA R2A, and NMDA R2C, following their transient co-expression in HEK 293 cells. A comparison of the pharmacological specificity between these cloned receptors with NMDA receptors expressed in the adult mouse cerebellum shows a high degree of correlation (r = 0.992), thus suggesting that in the native system, the three subunits coexist in an as yet unknown ratio. This co-association is consistent with in situ hybridization studies which show the co-localization of NMDA R1, NMDA R2A, and NMDA R2C mRNAs in adult cerebellar granule cells (29). The results substantiate the observations of Wafford et al. (7) which suggested a preferential co-assembly of the NMDA R1/2A/2C subunits expressed in Xenopus oocytes. Furthermore, they are in agreement with those of Sheng et al. (8) where the co-association of NMDA R1, 2A, and 2B subunits in native receptors was inferred from immunoprecipitation studies with subunit-selective antibodies.

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REFERENCES