Identification of amino acid residues of the NR2A subunit that control glutamate potency in recombinant NR1/NR2A NMDA receptors.

Philip E. Chen B.Sc. (Hons).

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Wellcome Laboratory for Molecular Pharmacology
Department of Pharmacology
University College London

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Abstract.

The NMDA type of glutamate receptor requires both glycine and glutamate to activate it efficiently. The receptor is thought to be an oligomer of two types of subunit, NR1 and NR2. Site-directed mutagenesis has shown that glycine potency is controlled by residues located in two areas on the NR1 subunit, one N-terminal of M1 and the other C-terminal of M3 (termed the S1 and S2 domains respectively). To test the hypothesis that the glutamate site exists on the NR2 subunits, the role of amino acid residues in similar areas on the NR2A subunit were investigated. These areas show homology with the ligand binding domains of bacterial periplasmic amino acid binding proteins.

Two mutations caused an increase of glutamate $EC_{50}$ by at least two orders of magnitude, (H466A and G669A) and one (T671A) by three orders, compared with wild-type. In contrast, glycine $EC_{50}$s did not differ by more than 2-fold compared with the wild-type. Schild analysis was used to measure the binding of the competitive NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid (APV) on the most 'shifted' mutant (T671A). The slope of the Schild plot for the mutant receptor did not differ greatly from unity, $0.94 \pm 0.16$ (mean $\pm$ S.D.M), which is consistent with APV being a competitive antagonist on the mutant receptor. However the affinity of the receptor for APV ($K_B = 321 \pm 30$ µM) was 255-fold less than wild-type ($K_B = 1.26 \pm 0.07$ µM) (means $\pm$ S.D.M).

The large reduction of glutamate potency, together with unchanged Hill slope, and no gross reduction in maximum response, suggests that residues contributing to glutamate binding are located on the NR2A subunit. This is supported by the much-reduced affinity for APV in the T671A mutant. Such evidence suggests that this residue is important for the binding of glutamate to NR1/NR2A receptors.

In a second project, transgenic founder mice were generated by pronuclear microinjection containing a 8 kb 5'UT fragment from the mouse NR1 gene driving the expression of the tetracycline-sensitive transactivator (tTA). This mouse may be useful for the study of neuronal-specific inducible gene expression in the future.
Acknowledgements.

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And finally, many thanks to my family and friends for their support over the last three years.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>3-hydroxy-5- methylisoxazolopropionic acid.</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate.</td>
</tr>
<tr>
<td>APV</td>
<td>2-amino-5-phosphonopentanoic acid.</td>
</tr>
<tr>
<td>7CK</td>
<td>7-chlorokynurenate.</td>
</tr>
<tr>
<td>CPP</td>
<td>3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid.</td>
</tr>
<tr>
<td>DCKA</td>
<td>5,7-diCl-KYN</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate.</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid.</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid (kainate).</td>
</tr>
<tr>
<td>MK-801</td>
<td>(+)-5-methyl-10, 11-dihydro-5H-dibenzo[α, d]cyclohepten-5, 10-imine.</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulphonic acid.</td>
</tr>
<tr>
<td>NFR</td>
<td>normal frog ringer.</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid.</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine.</td>
</tr>
<tr>
<td>PCP</td>
<td>phencyclidine.</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol.</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate.</td>
</tr>
</tbody>
</table>
SDM standard deviation of the mean, also known as standard error of the mean (SEM).

TEMED NNN'N'-Tetramethylene diamine.

TPA 12-O-tetradecaboylphorbol 13-acetate.
1. Introduction.

1.1. Glutamate receptors in the central nervous system.

Glutamate receptors play a major role in fast synaptic transmission in the central nervous system. They participate in several physiological and pathological processes that occur in the brain. This includes plastic changes in the strength of synaptic transmission, which are thought to underlie the basis of learning and memory. However, overexcitation of glutamate receptors can cause pathological situations such as neuronal cell death. Glutamate mediated neuronal toxicity is thought to be one of the basic mechanisms for neuronal cell death in neuronal degenerative diseases.

1.1.1. Types of glutamate receptors.

Glutamate activated receptors can be divided into two groups, fast acting ionotropic receptors and the slower acting metabotropic glutamate receptors (mGluR) which are coupled to G-proteins and mediate function through intracellular secondary messengers.

Ionotropic glutamate receptors have been divided into subtypes based upon the pharmacological selectivity of various synthetic agents. In the ionotropic family, three subtypes exist, the non-NMDA subtypes, AMPA and kainate and the NMDA subtype. However, the pharmacological distinction between non-NMDA receptor subtypes is not well defined because both AMPA and kainate receptors can be activated by kainate. AMPA receptors have been well characterised and they are thought to mediate fast synaptic transmission in vivo. However because of the non-selective nature of kainate it has been difficult to study native kainate receptor mediated responses in isolation.
1.1.2. Properties of the NMDA-type glutamate receptors.

NMDA is a synthetic analogue of the structure of aspartic acid which selectively activated one type of glutamate receptor (Watkins, 1962). These receptors were named 'NMDA' type receptors and have a number of unique properties that contribute to their physiological role as a coincidence detector for both pre and postsynaptic activity. Firstly, the ion channel is blocked by Mg\(^{2+}\) at normal resting potentials of the cell (-70 mV), this block can be relieved by depolarisation of the postsynaptic membrane (Nowak et al., 1984). Secondly, the cation-selective channel is permeable to Ca\(^{2+}\) unlike most other glutamate receptors. Thus the simultaneous (coincidence) presence of agonist and postsynaptic depolarisation is required for channel opening which results in a specific signal entering the cell i.e. Ca\(^{2+}\) influx. For efficient gating of the ion channel, not only does the receptor require glutamate as an agonist but also glycine as a co-agonist (Johnson and Ascher, 1987). Evidence has shown that the glutamate and glycine sites are separate and that two molecules of glutamate and two molecules of glycine are required for efficient gating of the receptor (Benveniste and Mayer, 1991).

NMDA receptors can be blocked by competitive antagonists such as APV (Davies et al., 1981) and noncompetitive ion channel blockers such as MK-801, ketamine and PCP.

1.1.3. Molecular diversity of ionotropic glutamate receptors.

Expression cloning in *Xenopus* oocytes revealed the cDNA for the first glutamate receptor subunit GluR1 (Hollmann et al., 1989). So far at least fourteen glutamate receptor subunits have been cloned, of these eight are non-NMDA receptor subunits and six are NMDA receptor subunits. Non-NMDA receptors can be homomeric or heteromeric oligomers composed of different subunits which give distinct functional properties to the channels. There are several 'orphan' subunits which have been cloned which are not activated by glutamate (or known agonists) such
as the GluR δ1 and 2 (Lomeli et al., 1993; Yamazaki et al., 1992a). Recently knockouts of these subunits in mice have revealed possible roles of these subunits in the brain. Knockouts of the GluRδ2 subunit impairs Purkinje cell synapse formation as well as motor co-ordination in mice (Kashiwabuchi et al., 1995). A mutation in the GluR δ2 subunit is responsible for cerebellar cell death in the Lurcher mouse mutant (Zuo et al., 1997).

<table>
<thead>
<tr>
<th>AMPA</th>
<th>KA</th>
<th>Non-NMDA</th>
<th>NMDA</th>
<th>Orphan subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat</td>
<td>mouse</td>
<td>GluR1/A</td>
<td>α1</td>
<td>GluR5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluR6</td>
<td>β2</td>
<td>GluR7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GluR2/B</td>
<td>α2</td>
<td>KA-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>KA-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GluR3/C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GluR4/D</td>
</tr>
</tbody>
</table>

Table 1. Cloned ionotropic glutamate receptor subunits divided into non-NMDA, NMDA families and orphan subunits.
1.2. Structure-function characteristics of the NMDA receptors-
molecular biology and function.

1.2.1. Cloning of the NMDAR subunits.

Expression cloning in *Xenopus* oocytes was also used to isolate the NR1 subunit cDNA from a size selected (3-5 kb) rat forebrain cDNA library (Moriyoshi et al., 1991). Subsequently, the four NR2 subunits were obtained from rat brain, NR2A-C (Monyer et al., 1992) and NR2D (Ishii et al., 1993). The respective mouse NMDAR subunits were named £1 and e1-e4 (Ikeda et al., 1992; Kutsuwada et al., 1992; Meguro et al., 1992; Yamazaki et al., 1992b).

The mature NR1-la subunit is composed of 920 amino acids and the protein was predicted to have a molecular mass of 103 kDa. The NR1 subunit exists as 8 splice variants (Durand et al., 1992; Hollmann et al., 1993; Nakanishi et al., 1992; Sugihara et al., 1992). They are created (compared to NR1-la) by one or more combinations of three different splicing events (Figure 1.1): (i) The insertion of an extra exon (63 bp-exon 5) into the N-terminal domain (dividing the splice variants into 'a' and 'b' forms). (ii) Deletion of exon 21 (111 bp) from the C-terminal domain after M4. (iii) Alternative splicing in exon 22 deleting 356 bp and generating a new stop codon. The most common variant, NR1-la has been estimated to have a relative abundance of 67 % in adult rat brain (Sugihara et al., 1992).

The NR2A-D subunits have amino acid sequence lengths of 1445, 1456, 1220 and 1296 respectively. They have predicted molecular masses of 163, 163, 134 and 141 kDa respectively. Only the NR2D subunit is known to exist in two isoforms. The NR2A and NR2B subunits have relatively large C-terminal regions (627 and 644 amino acid residues) compared to the other NMDAR subunits but also with the non-NMDA receptor subunits. The large NR2 C-terminal, although not necessary for the formation of functional channels (Kohr and Seeburg, 1996), may be important for intracellular signalling. Overall sequence homology is high between the NR2 subunits.
(40-50 %). The NR2A and NR2B subunits have an overall amino acid sequence identity of about 70 % while the NR2C and NR2D subunits have a sequence identity of 55 % (Monyer et al., 1992). However when compared to the NR1 subunit, the identity is reduced to 18 %. This is similar to the identity between NMDAR subunits and the other glutamate receptor subunits.

An 'NR1 like' subunit has been cloned and has been renamed NR3 (Ciabarra et al., 1995; Sucher et al., 1995). It shares 27 % identity with the NR1 and NR2 subunits.

Human NMDA subunit cDNAs have been cloned and have high sequence homology with rodent receptors (Karp et al., 1993; Le Bourdelles et al., 1994). Human NR1 has 99 % amino acid residue sequence identity with rat NR1 while NR2A has 95 % sequence identity with its rat counterpart (Le Bourdelles et al., 1994). The largest divergence between rat and human NR2 subunits is within the C-terminal region.
Figure 1.1. Profiles of NR1 splice variants and NR2 subunits. Insertions and alternative splicing are shown for each NR1 splice variant. The box above the 'b' NR1 variants indicates the location of the alternatively spliced exon 5 in the N-terminal region. Putative signal peptides and internal hydrophobic regions are shown as patterned and filled boxes. From Hollman et al., (1994) and Mori et al., (1995).
1.2.2. NMDAR channels are heteromeric.

When the NR1 subunit was expressed with any of the four NR2 subunits very robust glutamate-evoked inward currents were observed in the presence of glycine (Ishii et al., 1993; Monyer et al., 1992). Binding studies have shown that channel blocking agents such as MK-801 were bound with high affinity in cell lines expressing both NR1 and NR2 subunits together (Lynch et al., 1994; Lynch et al., 1995).

Other pieces of evidence suggest that native NMDA receptors are heterooligomers. Immunoprecipitation studies (Sheng et al., 1994) have shown that anti-NR1 subunit antibodies immunoprecipitated not only a 120 kDa protein but also some NR2A and NR2B protein as well from rat cerebral cortex. Quantitative immunoblot analysis has shown that the majority of NMDA receptor complexes in adult rat cerebral cortex are composed of NR1/NR2A/NR2B subunits (Luo et al., 1997). Evidence for this combination of NMDA receptor subunits has also been shown for NMDA receptors from rat forebrain (Chazot and Stephenson, 1997). Biochemical evidence coupled with MK-801 binding data has also suggested that NR2A and NR2C may form a complex with NR1 subunits (Chazot et al., 1994). Recent work has also shown the association of NR1/NR2A/NR2D and NR1/NR2B/NR2D subunits in the cortex and thalamus (Dunah et al., 1998). Furthermore, functional evidence for the assembly of recombinant NR1/NR2A/NR2D receptors has been shown by single-channel studies (Cheffings and Colquhoun, 1999).

In addition to heteromeric expression, NR1 subunits were previously though to form 'homomeric channels' when expressed alone in *Xenopus* oocytes (Moriyoshi et al., 1991), however the current responses were very small (~ 10 nA). The currents could be evoked by glutamate in the presence of glycine or by NMDA in the same conditions. However homomeric NR1 responses were not seen when expressed in mammalian HEK 293 cells (Monyer et al., 1992). It was shown in COS cells that NR1 only expressing cell lines did not cause an increase in intracellular calcium concentration.
in Fura-2 loaded cells upon application of NMDA or glutamate (Ishmael et al., 1996). Additionally, NMDA receptor channel blocking agents such as MK-801 were not detected in NR1 subunit expressing cells (Grimwood et al., 1995; Ishmael et al., 1996; Lynch et al., 1994). Cell surface expression of the human NR1α subunit was only found in cell lines co-transfected with NR2A (McIlhinney et al., 1996). However, certain C-terminal NR1 splice variants (4a and 4b forms—see section 1.2.1) were detected on the cell surface when expressed alone in fibroblasts (Okabe et al., 1999).

1.2.3. General topology of glutamate receptors.

The present topological model for glutamate receptors suggest that the protein contains three transmembrane domains (M1, M3 and M4) with M2 forming an inverted loop in the membrane and with the N-terminal and C-terminal regions located extracellularly and intracellularly respectively (Figure 1.2) (Wo and Oswald, 1995b). The inverted loop structure for M2 was proposed after N-glycosylation analysis of GluR1 receptors showed that the M2 region entered the membrane but did not cross it (Hollmann et al., 1994). This conclusion was also reached after analysing the protease sensitivity of epitope tagged GluR3 receptors to identify the proposed topology of the peptide (Bennett and Dingledine, 1995). Placement of epitopes surrounding M2 revealed that the putative transmembrane domain was a re-entrant loop. The structure of the M2 domain is analogous to the H5 domain between S5 and S6 which acts as an inverted re-entrant loop lining the pore of voltage-gated K⁺ channels (Bennett and Dingledine, 1995; Hollmann et al., 1994).

This model has been confirmed for NMDA receptors by recent work using cysteine-substitution methods to map residues contributing to the channel pore. Amino acid residues in the M2 domains of the NR1 and NR2C subunits were substituted with cysteine residues. Residues lining the channel pore were identified by their accessibility to and thus interaction with sulphydryl-specific reagents (Kuner et al., 1996). The M2 region was shown to form a channel lining loop originating and
terminating in the cytoplasmic side of the channel. Furthermore, a model was proposed for the location of the M2 residues with respect to the channel pore.

Previously glutamate receptors were predicted to have a large extracellular N-terminal region before the four putative transmembrane regions (M1-M4) and an extracellular C-terminal region. This was based upon the structures predicted by hydrophobicity studies of the amino acid sequence of other ligand-gated ion channels. This view changed due to contradicting evidence about the predicted locations of these regions. Firstly, phosphopeptide analysis had shown that phosphorylation sites exist in the C-terminal region of the NR1 subunit (Tingley et al., 1993). Additionally, the C-terminal region of the ε2 subunit controls the activation of the ε2/ζ1 ion channel by the PKC activator, TPA, suggesting that the C-terminal is intracellular (Mori et al., 1993). Secondly, site-directed mutagenesis studies showed that the loop between M3 and M4 was extracellular and not intracellular. This is because this region is believed to be a location involved with agonist binding (Anson et al., 1998; Kuryatov et al., 1994; Laube et al., 1997; Stern Bach et al., 1994; Williams et al., 1996). Structural studies of the glutamate binding site in complex with kainate, have shown that the region between M3 and M4 does form an agonist binding pocket (Armstrong et al., 1998). Finally N-glycosylation analysis of goldfish kainate binding proteins and GluR1 receptors revealed that the loop between M3 and M4 was extracellular (Hollmann et al., 1994; Wo and Oswald, 1994; Wo and Oswald, 1995a).
Figure 1.2. Proposed transmembrane topology for glutamate receptors. Membrane-spanning regions 1, 3 and 4 are shown crossing the lipid bilayer. M2 is shown as a re-entrant loop entering and leaving the lipid bilayer on the cytoplasmic side. N- and C-terminal regions are located extracellularly and intracellularly respectively.
1.2.4. Stoichiometry.

The exact stoichiometry of NMDA receptors is unclear. Although most evidence suggests that two NR1 subunits are present in the receptor. Coexpression of wildtype and mutant NR1 subunits has been used to produce intermediate conductance levels (Behe et al., 1995). The number of intermediate conductance levels suggested that the NR1 exists as 2 copies in the NMDA receptor complex. But it was implied that the number of NR2 subunits must be more than two. However, others using the same technique found evidence to suggest that the NR1 exists as 3 copies instead, proposing a pentameric structure with two NR2 subunits (Premkumar and Auerbach, 1997). Biochemical evidence has shown that by coexpressing FLAG- and c-Myc epitope-tagged NR2B with NR1 and NR2A subunits in HEK293 cells, three NR2 subunits were found to co-associate within the same receptor complex, which would suggest that the receptor is at least pentameric (assuming that there are at least two NR1 subunits) (Hawkins et al., 1999).

The case for a tetrameric receptor has been shown by exploiting the differences in dose-response relationships between wildtype and mutant NMDA receptor channels with reduced glutamate and glycine potencies. Fitted dose-response curves suggested that two NR1 and two NR2 subunits were needed to form the NR1/NR2B receptor (Laube et al., 1998).

The same inconsistently exists for non-NMDA receptor stoichiometry. Some studies have suggested that the non-NMDA receptors are tetrameric. Experiments using chimeric GluR6/GluR3 channels have counted the number of glutamate binding sites (and thus subunits present in the receptor) by observing the different electrophysiological states the channel passes through as more of the agonist is bound. These results suggested that the glutamate receptor is a tetramer rather than a pentamer (Rosenmund et al., 1998). However, others have provided evidence that homomeric GluR1 receptors are pentameric (Ferrer Montiel and Montal, 1996).
1.2.5. Structure and function of the channel-lining regions.

Site-directed mutagenesis has shown that an arginine at position 586 in the M2 domain of the GluR2 subunit controls Ca\textsuperscript{2+} permeability of the AMPA receptor (Hume et al., 1991; Verdoorn et al., 1991). Incorporation of the GluR2 subunit within the receptor complex results in Ca\textsuperscript{2+} impermeability of the AMPA receptor. The same position in the NMDAR subunits is occupied by an asparagine residue (N) instead. Replacement of this asparagine with a glutamine in the NR1 subunit leads to a reduction in Ca\textsuperscript{2+} permeability. Moreover, the Ca\textsuperscript{2+} permeability is abolished when an arginine residue (R) is placed in this position (Burnashev et al., 1992; Mori et al., 1992). Replacement of the asparagine residue with a glutamine, in the NR2A/NR2B subunits, resulted in a partial reduction in Mg\textsuperscript{2+} block (Burnashev et al., 1992; Mori et al., 1992). This N-site (or Q/R/N-site) is thought to be located at the tip of the M2 inverted loop described earlier (Kuner et al., 1996). Interestingly the NR1 and NR2C M2 domains examined in this study were found to have different patterns of channel lining residues. This may account for the unequal contribution of the N-site between NR1 and NR2 subunits to the Ca\textsuperscript{2+} permeability and Mg\textsuperscript{2+} block of the NMDA receptor (Burnashev et al., 1992).

It has been suggested that the effect of the N-site is to constrict the permeation pathway of the channel. The cross-sectional diameter of the constriction has been estimated to be 0.55 nm by measuring the relative permeability of differently sized organic cations (Wollmuth et al., 1996). Mutation of the N-site on the NR1 subunit to a glycine and the N+1 site (C-terminal side) on the NR2A subunit, produced an ion channel with an increased pore size of 0.87 nm. Thus the asparagine at the N-site of the NR1 subunit and the N+1 site of the NR2A subunit contribute to the narrow constriction of the channel.

Recent work has identified that a tryptophan at position 607 within the M2 domain of the NR2B subunit has a role in controlling Mg\textsuperscript{2+} block in recombinant NR1/NR2A/B channels (Williams et al., 1998). Replacement of this tryptophan (W)
with a leucine (L) residue resulted in a channel with no Mg\textsuperscript{2+} block and an increased permeability to extracellular Mg\textsuperscript{2+}. It was suggested that this tryptophan is involved in binding divalent cations within the channel and acts with the asparagine residues from the NR1 and NR2 subunits to form a selective filter against Mg\textsuperscript{2+}. Further work has been carried out on the N-site and the N+1 site, suggesting that intracellular Mg\textsuperscript{2+} interacts with the asparagine at the N-site from the NR1 subunit while extracellular Mg\textsuperscript{2+} interacts with the N+1 asparagine residues from the NR2A subunits (Wollmuth et al., 1998a; Wollmuth et al., 1998b).

The NR2 subunits contribute to the sensitivity of the NR1/NR2 ion channels to Mg\textsuperscript{2+} block. For instance, ion channels formed from the NR1-NR2A/B combination are more sensitive to Mg\textsuperscript{2+} block than NR1-NR2C/D ion channels (Kutsuwada et al., 1992; Monyer et al., 1992). At physiological concentrations of extracellular Mg\textsuperscript{2+} (1 mM) NR1/NR2A and NR1/NR2B ion channels are characterised by a stronger voltage-sensitive block than the combinations containing NR2C and NR2D subunits (Monyer et al., 1994). The multiple structural elements responsible for NR2 subunit specificity for Mg\textsuperscript{2+} block has been pinpointed to regions within M1, between M2-3 and M4 of the NR2 subunits (Kuner and Schoepfer, 1996).
Figure 1.3. Alignments of the M2 regions of the AMPA and NMDA receptor subunits. Boxed residues show the position of the Q/R/N site. A glutamine residue (Q) is present in GluR1, 3 and 4 whereas this is replaced by an arginine (R) in GluR2. In NMDAR subunits this position is taken up by an asparagine (N).
1.2.6. **Pharmacological diversity of recombinant NMDA receptors.**

Experiments using the heterologous expression of recombinant NMDA receptor subunits have shown the pharmacological diversity of NMDA receptors depending upon the type of NR2 subunit in the receptor composition. For the mouse NR1/NR2 receptors expressed in *Xenopus* oocytes the glutamate $EC_{50}$ values for $\varepsilon 1/\zeta 1$, $\varepsilon 2/\zeta 1$, $\varepsilon 3/\zeta 1$ and $\varepsilon 4/\zeta 1$ combinations were 1.7, 0.8, 0.7 and 0.4 µM respectively. In the same order glycine $EC_{50}$ values were 2.1, 0.3, 0.2 and 0.09 µM (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992). In terms of antagonist sensitivity, the $\varepsilon 1/\zeta 1$ combination was found to be the most sensitive to the glutamate site antagonist, APV and $\varepsilon 3/\zeta 1$ combination was the most sensitive to the competitive glycine site antagonist, 7CK. The $\varepsilon 4/\zeta 1$ combination was not only the most sensitive to both glutamate and glycine but also the least sensitive to either APV or 7CK (Ikeda *et al.*, 1992; Kutsuwada *et al.*, 1992).

For the corresponding rat subunits the rank order of glutamate and glycine potencies are similar to those found for the mouse subunits (see Table 2). Antagonist sensitivities follow a similar trend to those found for the mouse heteromeric combinations.

<table>
<thead>
<tr>
<th>Receptor Composition</th>
<th>Glutamate $EC_{50}$ (µM)</th>
<th>Glycine $EC_{50}$ (µM)</th>
<th>APV $IC_{50}$ (µM)</th>
<th>7CK $IC_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1/NR2A</td>
<td>~ 3.0</td>
<td>~ 2.0</td>
<td>2.5</td>
<td>0.7</td>
</tr>
<tr>
<td>NR1/NR2B</td>
<td>~ 1.5</td>
<td>~0.5</td>
<td>4.7</td>
<td>0.62</td>
</tr>
<tr>
<td>NR1/NR2C</td>
<td>1.0</td>
<td>N/A</td>
<td>13</td>
<td>3.2</td>
</tr>
<tr>
<td>NR1/NR2D</td>
<td>0.45</td>
<td>0.1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The NR1/NR2A and NR1/NR2B combinations are more sensitive to divalents such as Mg$^{2+}$ (Mg$^{2+}$ IC$_{50}$ ~ 2 μM) with the NR1/NR2C and NR1/NR2D receptors having Mg$^{2+}$ IC$_{50}$ values in the range of 10-15 μM (Kuner and Schoepfer, 1996; Monyer et al., 1994). A similar trend among the heteromeric NR1/NR2 combinations is seen with Zn$^{2+}$ sensitivity (Traynelis et al., 1998).

NR1/NR2A and NR1/NR2B combinations ($K_D = 4$-$5$ nM) are also more sensitive to the non-competitive ion channel blocker, MK-801 than NR1/NR2C/D combinations ($K_D = 150$ nM) (Laurie and Seeburg, 1994a). However, sensitivity to other drugs such as ketamine and PCP is similar among all four combinations (Ikeda et al., 1992; Kutsuwada et al., 1992). There are few subtype specific NMDA antagonists (limiting the study of different NR1/NR2 subtypes in native systems), the most notable exception being the pheylethanolamine, ifenprodil. Ifenprodil is a non-competitive antagonist with a high selectivity for NR2B containing receptors over NR2A containing receptors (Williams, 1993). Ifenprodil is thought to antagonise NMDA receptor function by increasing its sensitivity to proton inhibition (Mott et al., 1998).

In contrast, NR1a subunits expressed alone in Xenopus oocytes were found to have glutamate and glycine EC$_{50}$ values of 4.0 μM and 0.64 μM respectively (Hollmann et al., 1993). However, NR1 splice variants containing exon 5 had an approximately 4-fold lower glutamate potency (EC$_{50}$ ~ 12.0 μM) but a similar glycine potency (EC$_{50}$ ~ 0.75 μM) to NR1a channels. NR1 C-terminal splice variants had similar glutamate and glycine potencies to the NR1a form (Durand et al., 1993).

1.2.7. NMDA receptor competitive antagonists.

These antagonists act either at the glutamate or glycine binding sites. The classical glutamate site antagonists are based upon the structure of phosphono analogues of amino acids, such as D-APV and D-AP7 (Evans et al., 1982). New antagonists based upon these structures are still being synthesised, such as SDZ 220-
040, where a biphenyl ring is inserted into the carbon chain of AP7 (Urwyler et al., 1996) and CGP-19755 (Laurie and Seeburg, 1994a). Other antagonists have been discovered such as, D-CPP and its unsaturated analogue, D-CPPene, which are based upon a series of 4-substituted piperazine-2-carboxylic acids (Aebischer et al., 1989; Davies et al., 1986). The original glycine site antagonists were based upon the structure of kynurenic acid, such as the halogen substituted, 7-CK and the more potent variants made by substituting the 5- and 7- positions with iodo- and chloro- groups, such as 5,7-DCKA and 5-iodo-7-chlorokynurenic acid (L-683,344) (Danysz and Parsons, 1998). Other potent glycine site antagonists include the 2-carboxyindoles, such as MDL-105,519 and the 2-carboxytetrahydroquinoline, L-689,560, which happens to be one of the most potent NMDA receptors antagonists discovered so far.

1.2.8. Single-channel studies.

In the presence of Mg\(^{2+}\), the single-channel recordings are characterised by bursts of short openings separated by brief closures (Ascher and Nowak, 1988). The duration of these short openings decreased with increasing Mg\(^{2+}\) concentration, but unaffected the duration of the brief closures. Depolarisation increased or decreased the duration of short openings and closures respectively. This flickering channel activity is increased with higher Mg\(^{2+}\) concentrations and is abolished by depolarisation. These bursts are though to be caused by the blocking/unblocking of the channel by Mg\(^{2+}\).

In the absence of Mg\(^{2+}\), single channel recordings from adult rat hippocampal CA1 cells revealed that upon application of low glutamate concentrations in the absence of divalents, a complex cluster of channel openings from a single activation of the NMDA receptor were observed (Gibb and Colquhoun, 1992). Activation of the receptor produced several bursts which were grouped together as a long cluster, unlike those seen for the nicotinic acetylcholine receptor where an activation was mainly composed of a single burst of closely spaced openings (Colquhoun and Sakmann, 1985). Distribution of the channel shut times contained a number of relatively long
closed periods, the durations of these were not influenced by either glutamate or glycine concentrations. It was suggested that unlike other ligand gated ion channels with similar open times, the NMDA receptor channel is closed for a significant period of time while the agonist is still bound to the receptor. Therefore, glutamate can be characterised as acting as a partial agonist when activating the NMDA receptor (Gibb and Colquhoun, 1992).

Studies on native NMDA receptors have shown that they appear as two conductance levels, 50 pS openings and brief 40 pS sublevels with similar mean lifetimes and frequencies (Ascher and Nowak, 1988; Gibb and Colquhoun, 1992; Nowak et al., 1984). In addition, a lower conductance state was also observed in cerebellar neurons of 38/18 pS (Cull-Candy and Usowicz, 1987). In cerebellar granule cells there is diversity in the single channel properties of the NMDA receptor during development. Before P13, most openings were seen at the 50/40 pS conductance state, however by P19 this changed, with a majority of conductances seen at the lower 33/20 pS state (Farrant et al., 1994).

Conductances in the 50/40 pS range have been observed from *Xenopus* oocytes and HEK 293 cells expressing recombinant NMDA receptor NR1/NR2A or NR2B subunits (Stern et al., 1992; Stern et al., 1994). Studies on the NR1/NR2C and NR1/NR2D combinations revealed lower conductance levels of 35 and 20 pS of similar duration (Stern et al., 1992; Wyllie et al., 1996). This division between NR1/NR2A or NR2B and NR1/NR2C or NR2D channels mirrors the similarities between the two groups of NR2 subunits, in terms of amino acid sequence identity and Mg$^{2+}$ sensitivity (see 1.2.6.). Introduction of the N598R mutation in the NR1 subunit which controls voltage-dependent Mg$^{2+}$ block (when expressed with NR2A) produces very small conductance levels of 2.6 pS (main level) and 1.2 pS (sublevel) (Behe et al., 1995).

Unlike the other NR1/NR2 heteromeric pairings, NR1/NR2D channels show temporal asymmetry in the frequency of transitions between the main and sublevel conductance states (Wyllie et al., 1996).
It has been shown that in terms of conductance, single channel recordings from NR1/NR2B ion channels are similar to recordings from hippocampal CA1 cells while the NR1/NR2C combination resembles the recordings seen from immature large cerebellar neurones from P17-19 rats (Stern et al., 1992).

1.2.9. Kinetics.

The activation and deactivation kinetics for native NMDA receptors were found to be much slower than that for AMPA receptors. NMDA-receptor mediated currents elicited by 5 ms pulses of 100 μM glutamate were found to have a 10-90 % rise time of about 10 ms and a decay that could be fitted by two exponentials (90 ms and 260-600 ms) (Lester et al., 1990). The decay of NMDA evoked currents were shown to be dependent upon firstly the unbinding rate of the agonist from the receptors and thus the affinity of the agonist (glutamate) but also the rate of desensitisation of the NMDA receptor itself (Lester et al., 1990; Lester and Jahr, 1992). In recombinant NR1/NR2 receptors expressed in HEK 293 cells, the decay time constants estimated from 300 ms pulses of glutamate were found to vary depending upon the type of NR2 subunit expressed with the NR1 subunit. The NR1-NR2B and NR1-NR2C combinations had time constants of about 400 ms, while the NR1-NR2A ion channels had faster decay time constants of 120 ms. The NR1-NR2D combination had a much longer time constant of about 4800 ms (Monyer et al., 1994; Wyllie et al., 1998).

The excitatory postsynaptic currents (EPSCs) seen at excitatory synapses in the CNS are made up of AMPA receptor and NMDA receptor mediated EPSCs. NMDA receptor mediated EPSCs have slower rise and decay times than AMPA-EPSCs (rise times, 200 μs with decay time constants, 1-3 ms (Mosbacher et al., 1994)). This difference between the decay times has been shown, so far to be due to different affinities of the receptors for glutamate. The NMDA receptor has a higher affinity than AMPA receptors for glutamate, resulting in prolonged binding during which the channel can be opened repeatedly (Lester et al., 1990; Lester and Jahr, 1992). In the
The pulse of glutamate released at the synapse is enough to activate both AMPA and NMDA type receptors. The difference in activation and decay kinetics of the two receptors are important for the role of NMDA receptors in excitatory synaptic transmission (McBain and Mayer, 1994).

1.2.10. Desensitisation.

NMDA receptors are characterised by three types of desensitisation. Glycine-dependent desensitisation is due to the allosteric reduction of glycine affinity by glutamate binding (Mayer et al., 1989). Glycine-independent desensitisation which causes a time-dependent rundown in NMDA receptor mediated current responses (Sather et al., 1990) and Ca\(^{2+}\)-dependent inactivation which is due to increases in intracellular Ca\(^{2+}\) (Legendre et al., 1993).

Recent work has shown that a region in the N-terminal domain of the NR2A subunit has some role in glycine-independent desensitisation of the NMDA receptor (Krupp et al., 1998; Villarroel et al., 1998). Chimeras between NR2A and NR2C subunits showed that glycine-independent desensitisation is controlled by 2 areas (one just preceding M1 and a 190 amino acid residue region further N-terminal of the former) in the N-terminal region from the NR2A subunit. Mutations of amino acid residues at positions A555 and S556 in the NR2A subunit caused slight reductions in the extent of the desensitisation (but not a total loss) suggesting that these residues have a role in this type of desensitisation.

Ca\(^{2+}\)-dependent inactivation has been shown to be controlled by the C-terminal region of the NR1 subunit (C0 exon) and is due to the binding of calmodulin to the C0 exon (Ehlers et al., 1996). Ca\(^{2+}\)-sensitive calmodulin displaces the cytoskeletal protein, \(\alpha\)-actinin from the C0 exon, disrupting the normal interaction of the C-terminal region of the NMDA receptor with the cytoskeleton (Krupp et al., 1999; Zhang et al., 1999).
1998). This may act as a negative feedback mechanism to limit the amounts of Ca$^{2+}$ entering the cell.

1.2.11. Phosphorylation and modulation by other agents.

Original studies have shown that responses from different NR1 splice variants can be potentiated by the protein kinase C (PKC) activator, PMA (Durand et al., 1992). Phosphopeptide mapping revealed that this phosphorylation occurs at an exon in the C-terminal domain which is regulated by alternative splicing (Tingley et al., 1993). The NR1 splice variants may also regulate certain aspects of localisation of the receptor at the postsynaptic membrane. Expression of NR1 splice variants in fibroblasts showed that a C-terminal exon also regulates the subcellular distribution of the NR1 subunit, this can be disrupted by PKC phosphorylation within this exon (exon C1) (Ehlers et al., 1995). Interestingly, NR1 splice variants expressed in Xenopus oocytes lacking this C-terminal exon produced a greater phorbol ester-induced enhancement of NMDA-evoked currents (Durand et al., 1993; Durand et al., 1992). Furthermore, it was shown that protein kinase A also phosphorylates serine residues within this exon in transfected cells and in hippocampal slices (Tingley et al., 1997).

Work on heteromeric recombinant NMDA receptors showed that the responses from NR1a/NR2A or NR2B channels were potentiated by the PKC activator, TPA, but not the NR1a/NR2C or NR2D channels (Kutsuwada et al., 1992). This potentiation was sustained for up to 1 hr after treatment by NR1/NR2B currents. Chimeras generated between the NR2B and NR2C subunits showed that this phosphorylation is controlled by the C-terminal domains of the NR2B subunit (Mori et al., 1993). In contrast to the results reported by Tingley et al, (1993) expression of the NR1a splice variant (lacking the alternatively spliced C-terminal exon) with the NR2A subunit produced currents that could still be potentiated by the PKC activator, PMA (Sigel et al., 1994). Thus the molecular components underlying PKC-induced potentiation of NMDA-evoked currents remains to be clarified. On the single channel level,
phosphorylation by PKC has also been shown to reduce the voltage-dependent Mg$^{2+}$ block at native NMDA receptors and increase the probability of channel openings (Chen and Huang, 1992).

Dephosphorylation has also been shown to regulate NMDA channels as serine/threonine phosphatases can reduce the open probability of NMDA channels (Wang et al., 1994).

Tyrosine kinases and phosphatases regulate NMDA receptor activity as well (Wang and Salter, 1994). Biochemical evidence suggests that the NR2A and NR2B subunits from the synaptic membrane are tyrosine phosphorylated in vivo (Lau and Huganir, 1995). The NR2B subunit was found to be the major phosphorylated subunit in postsynaptic densities (Moon et al., 1994). Recombinant expression of NR1/NR2A subunits in HEK 293 cells has shown that glutamate currents can be enhanced by the tyrosine kinases src and fyn and that this phosphorylation occurs on the C-terminal region of the NR2A subunit (Kohr and Seeburg, 1996). However, current potentiation was not seen for heteromeric receptors containing the other NR2 subunits.

Other agents such as the polyamines can modulate NMDA receptor responses and this occurs in multiple ways (McBain and Mayer, 1994; Sucher et al., 1996). Spermine can potentiate the effect of glycine (glycine-dependent effect) and enhance NMDA receptor mediated currents at saturating glycine concentrations (glycine-independent effect). Additionally spermine can block the ion channel in a voltage-dependent manner. Spermine has specific glycine-independent potentiation on NMDA receptors containing NR1 splice variants without the exon 5 insertion and especially heteromeric combinations of NMDA receptors containing the NR1 and NR2B subunits (Williams et al., 1994). However, any effect of endogenous polyamines on NMDA receptors in vivo remains to be shown.

NMDA receptor mediated responses can be inhibited by protons within the physiological range of extracellular pH (Traynelis and Cull-Candy, 1990). This has been shown to be dependent upon the inclusion of exon 5 in NR1 splice variants but not the NR2 subunits (Traynelis et al., 1995).
Concentrations of Zn\(^{2+}\) > 10 \(\mu\)M can inhibit NMDA function in a voltage-dependent manner but this block is voltage independent at concentrations < 10 \(\mu\)M (McBain and Mayer, 1994). Recent studies have shown that the presence of exon 5 in NR1 splice variants can reduce the effect of Zn\(^{2+}\) inhibition similar to that found for the effects of protons (Traynelis et al., 1998). The application of spermine was found to reduce the Zn\(^{2+}\) inhibition as well. Thus protons, Zn\(^{2+}\) and polyamines may act at sites which share common structural elements on the NR1 subunit when modulating NMDA receptor function.

Ethanol at concentrations associated with intoxication in humans (5-50 mM) has been shown to inhibit NMDA receptor mediated responses. This reduction in the maximal response was found to be the strongest on NR1/NR2A NMDA receptor channels among the other NR1/NR2 subunit combinations (Kuner et al., 1993; Masood et al., 1994).

1.2.12. Distribution of NMDA receptor subunits in the developing and adult brain.

Using in situ hybridisation of NMDAR subunit mRNAs, the NR1 subunit was shown to be expressed ubiquitously at prominent levels throughout the adult rat brain (Akazawa et al., 1994; Monyer et al., 1994; Monyer et al., 1992; Moriyoshi et al., 1991). The strongest expression being in the cerebral cortex, hippocampus, cerebellum and the olfactory bulb. The NR2 subunits were found to be differentially expressed in the adult rat brain (Ishii et al., 1993; Monyer et al., 1994; Wenzel et al., 1997; Zhong et al., 1995). The NR2A mRNA is present in the forebrain, cerebellum and olfactory bulb, closely matching the distribution of NR1 mRNA (Ishii et al., 1993; Monyer et al., 1992). The NR2B subunit is more restricted and is mainly found in the forebrain, olfactory bulb and hippocampus while the NR2C is localised to the cerebellum (predominantly in the cerebellar granule layer) with weak signals in the olfactory bulb. The NR2D subunit was hardly seen at all in the cerebral cortical regions (Monyer et al., 1991).
1994) but weak signals were found in the lower brain stem regions, the glomerular
layer of the olfactory bulb, the hypothalamus, substantia nigra and the superior
colliculus (Ishii et al., 1993). Different expression patterns were also found for the
NR1 splice variants in the adult rat brain (Laurie and Seeburg, 1994b). Similar patterns
of expression were found for the mouse NMDA receptor subunits (Watanabe et al.,
1993; Watanabe et al., 1994a; Watanabe et al., 1994b).

During development the distributions of the NR1 and NR2 subunits undergo
specific temporal and spatial changes in the rodent brain (Monyer et al., 1994). At
embryonic stage E14, high levels for the NR1 (cortex, hippocampus and spinal cord)
and lower levels for the NR2B (hippocampus and spinal cord) and NR2D (spinal cord)
mRNAs were found but no NR2A nor NR2C were detected. At E17, the levels of the
NR2B and NR2D mRNAs were increased compared to E14 levels. At P0, the NR2B
mRNA was expressed mainly in the forebrain and hippocampus while the level of
NR2D expression peaks in the thalamus, hypothalamus and cortex. The NR2A and
NR2C transcripts appear after birth and are seen clearly by P7. NR2A mRNAs were
weakly (compared to NR2B levels) expressed in the hippocampus and the forebrain,
while the NR2C transcript was hardly detectable in the cerebellum (compared to NR2B
expression in the cerebellum at this stage) (Monyer et al., 1994; Wenzel et al., 1997;
Zhong et al., 1995).

Interestingly, by P12 the mRNA expression levels of the NR2B and NR2D
subunits begin to decrease and the NR2A (forebrain, hippocampus and cerebellum) and
NR2C mRNA levels (cerebellum) begin to increase (peaking during the third postnatal
week) (Wenzel et al., 1997). The levels of expression of NR1, NR2A and NR2B
subunits are reduced slightly in the brain when adulthood is reached (compared to P21
levels). The NR2A subunit emerges as the preponderant NR2 subunit being expressed
ubiquitously throughout the adult brain (Monyer et al., 1994; Wenzel et al., 1997).
The NR2B expression is restricted to the hippocampus and the forebrain while the
NR2D subunit almost disappears. Thus, during development the expression of the
main NR2 subunits switches from the NR2B and NR2D to the NR2A and NR2C variants.

This is also seen at the cellular level in cerebellar granule cells. High resolution emulsion autoradiography showed that there is a change in NR2 expression from the NR2B subunit to the cerebellar NR2C subunit as the postsynaptic brain develops (Monyer et al., 1994). This is supported by functional data where the single-channel properties of NMDA receptors in cerebellar granule cells change during development. After P19, the majority of 40/50 pS openings seen before P13 were switched to a minority and a majority of lower conductance openings (33/20 pS) seen instead (Farrant et al., 1994).

The different properties of NMDA receptor channels that are determined by the four NR2 subunits may allow the different regional and temporal expression of the NR2 subunits to adjust the properties of the NMDA receptor channels for specific roles during development and adulthood. NMDA receptor heterogeneity during development may occur by the replacement of one NR2 subunit by another in a certain region (e.g. NR2B by NR2A and NR2C in the cerebellum) or by the generation of different NMDA receptor subtypes (e.g. in cerebral cortex, where the ratio of NR2A:NR2B increases during development (Wenzel et al., 1997).

1.2.13. NR1/NR2D recombinant receptors—properties compared to other NR1/NR2 combinations.

NR1/NR2D containing receptors are intriguing because they exhibit a number of differing properties compared to NR1/NR2A or NR1/NR2B containing receptors. Their single channel properties differ from NR1/NR2A receptors, with longer deactivation times (see 1.2.9), longer receptor activations and a lower probability of the channel being open within an activation (for 96% of the supercluster duration the channel is closed). NR1/NR2D receptors have lower single-channel conductance levels than NR1/NR2A or NR2B receptors and show transition asymmetry for the frequency of transitions between main and sub-level conductance states and vice versa. Glutamate
and glycine are more potent on NR1/NR2D receptors than the other heteromeric combinations. Thus, insertion of the NR2D subunit into the NMDA receptor complex would modulate the function of predominantly expressing NR2A/NR2B neurons (e.g by producing NMDA receptor-mediated EPSCs lasting for several seconds).

However, the physiological role of the NR2D subunit is unclear. NR2D mRNA transcripts are found in cerebellar Purkinje neurons, which also possess single-channel conductances identical to recombinant NR1/NR2D receptors during the first week of postnatal development (Momiyama et al., 1996; Wyllie et al., 1996). Unfortunately, NMDA receptor-mediated synaptic currents were not seen in these cells. The NR2D knockout, unlike its NR1 and NR2B counterparts, survives to adulthood and exhibited no obvious histological abnormalities in the various brain regions and in the formation of whisker-related neuronal patterns. However during behavioural testing, the mice showed a reduction in spontaneous behavioural activity compared to wild-type littermates (Ikeda et al., 1995). At the other extreme, overexpressing the (normally expressed during embryonic development) NR2D subunit produced adult mice which had no deficits in spatial learning tasks but showed impaired Schaffer collateral CA1 LTP /LTD (Okabe et al., 1998). Further work has shown that overexpression of NR2D subunits in the mature brain can suppress epileptogenesis (Bengzon et al., 1999).

1.3. The physiological and pathological role of NMDA receptors.

As mentioned before several properties of the NMDA receptor allow it to play key roles in the physiological and pathological functions in the brain. They will be briefly summarised here.
1.3.1. Role in synaptic plasticity, memory and learning.

Long-term potentiation (LTP) has been proposed as a model for the activity-dependent changes in synaptic efficacy. This is thought to provide the physiological basis for learning and memory in the brain (Bliss and Collingridge, 1993). The properties of the NMDA receptor can explain the molecular basis for this mechanism. During a tetanic stimulation or depolarisation of the postsynaptic cell, the long-lasting depolarisation (possibly through the fast activating AMPA receptors) relieves the Mg$^{2+}$ block of the NMDA receptors. The pulse of glutamate released which has activated the AMPA receptors is also required to activate the NMDA receptors within the time course of the depolarisation generated. The opening of the NMDA channel allows Ca$^{2+}$ influx into the cell and triggers the intracellular processes which result in a change in synaptic efficacy. Thus, the NMDA receptor acts as a coincidence detector of the synchronous presence of glutamate and postsynaptic depolarisation. Evidence for the link between the NMDA receptor and its effect on synaptic strength and learning and memory has been provided by pharmacological methods and also through the analysis of NMDA subunit knockout mice.

*In vitro* and *in vivo* applications of the NMDA receptor antagonist, APV have shown that the induction of hippocampal CA1-LTP can be inhibited (Bliss and Collingridge, 1993). Additionally, behavioural studies of rats infused with APV showed an impairment in spatial learning (Davis *et al.*, 1992). Interestingly the infusion of the glycine site antagonist, 7CK had little effect on the induction of hippocampal LTP but caused severe sensorimotor impairment and deficit in spatial learning (Bannerman *et al.*, 1997).

Genetic knockouts of the NMDA subunits have been used to investigate the role of the NMDA receptor in synaptic plasticity. The mouse knockout of the NR2A-ε1 showed a reduction of the NMDA evoked current and reduced hippocampal LTP compared to wildtypes (Sakimura *et al.*, 1995). These mice also had a moderate impairment in spatial learning. Hippocampal CA1-restricted disruption of the NR1
gene has been done in mice using the Cre-lox system (Tsien et al., 1996b). Adult mice lacked NMDA receptor-mediated synaptic currents and LTP in CA1 synapses and showed impaired spatial learning.

NR2A/NR2C double knockout mice have been generated and show a lost of motor co-ordination highlighting the role of the NR2A and NR2C subunits in excitatory mossy fibre-granule cell synapses in the cerebellum and importance of this in motor coordination (Kadotani et al., 1996).

1.3.2. Role in pathological pain conditions.

The sensation of pain messages from the periphery can be exaggerated in pathological situations where the pain threshold has been lowered to the point that non-noxious stimuli can cause severe pain (hyperalgesia). This mechanism is through to occur by activity-dependent changes in the dorsal horn neurons receiving afferents (wind-up). NMDA receptors are thought to act as 'coincidence detectors' in the spinal cord causing plastic changes analogous to the effects seen in the hippocampus (Dickenson, 1990).

1.3.3. Role in neuronal cell death.

NMDA receptors have been implicated in glutamate excitotoxicity. In cases of anoxic insult or other traumatic injury to the brain, excessive activation of NMDA receptors causes intracellular Ca^{2+} levels to rise and overactivate Ca^{2+} dependent cytosolic mechanisms which lead to cell death (Choi, 1988; Choi and Rothman, 1990). Application of NMDA receptor antagonists prevent the associated cell death after an ischemic insult in cortical cultures (Choi et al., 1988). Two other pieces of evidence have highlighted the role of NMDA receptors in this process. Expression of NR1 and NR2A subunits in non-neuronal cell lines and *Xenopus* oocytes leads to cell death unless the culture medium contains an NMDA receptor antagonist (Anegawa et al.,
1995; Cik et al., 1993). Secondly, expression of Ca\textsuperscript{2+} impermeable NR1 subunits containing the N598R mutation causes a decrease in cell death (Boeckmann and Aizenman, 1996).

1.3.4. Role in survival after birth.

This has been shown by the disruption of the NR1 and NR2B gene in mice (Forrest et al., 1994; Kutsuwada et al., 1996; Li et al., 1994). These subunits are essential for neonatal life since homozygous NR1 and NR2B pups die at P0 probably due to cardiac/respiratory failure or an impaired suckling reflex. Recent evidence has revealed that generation of respiratory rhythms are normal in homozygous NR1 knockout pups suggesting that NMDA receptors are not important for the development of prenatal circuits controlling respiratory rhythms (Funk et al., 1997). Although no gross anatomical changes in the brain were observed in homozygous NR1 knockout pups, there was disruption in the whisker-related patterning (barrelettes) in the trigeminal brainstem nuclei (Li et al., 1994). This was also found for NR2B knockout mice suggesting that ion channels formed from both subunits are essential for postnatal survival and implicates NMDA receptors in the refinement of sensory pathways possibly by activity-dependent mechanisms.
1.4. Location of the agonist-binding site of NMDA receptors.

1.4.1. Structure-function studies of other glutamate receptors.

The structural analysis of the agonist-binding site of receptors has been generally hampered by the difficulty in obtaining soluble proteins from membrane receptors for X-ray crystallography (especially for NMDA receptors). Thus, most structural information has been elucidated through indirect methods. By analogy with nicotinic acetylcholine receptors it was suggested that the agonist binding region of AMPA-receptors may reside in a part of the N-terminal region (Uchino et al., 1992) conserved among GluR subunits. Mishina and coworkers showed that amino acid residues in the N-terminal region of GluR1 subunits caused large reductions in agonist potency in heteromeric mutant GluR1/GluR2 receptors. The change of a glutamic acid (E) to a lysine (K) at amino acid position 398 caused the glutamate potency to decrease by 100,000 fold. However, this was based upon an estimated plateau of the dose-response curve since this could not be elicited from the mutant. The Hill coefficient for this mutant was 0.3 compared to the wild-type value of 2.2, suggesting that some change in the gating mechanism of the channel may have occurred. Any change of the arginine (R) residue at position 481 produced a non-functional channel indicating the importance of this residue. The reduction in glutamate potency was at least 20-fold when a lysine residue (K) was changed to a glutamic acid (E) at position 445. However, AMPA potency was only reduced by 6-fold and kainate potency was unchanged, suggesting that other residues may determine the selectivity of the receptor for these agonists. A more comprehensive study of the role of this lysine at position 445 suggested that this residue may line the agonist binding pocket (Li et al., 1995). This study showed that the desensitisation of the mutant receptor was unchanged compared to wild-type but that the antagonist binding affinity was altered in the mutant channels.
Figure 1.4. Location of amino acid residues in the N-terminal region of GluR subunits causing large changes in glutamate potency (arrows). These residues are also conserved in the GluR5 and GluR6 subunits. Alignment from Paas et al., (1996).
Other work was based upon the limited amino acid sequence homology between bacterial periplasmic binding proteins and the N-terminal domains of mGluR receptors (O'Hara et al., 1993). This led to the suggestion that the binding sites of glutamate receptors may be based upon the binding pocket of these bacterial proteins. The residues contributing to the binding pocket in LAOBP (lysine/arginine/ornithine-binding protein) and histidine-binding proteins are known from X-ray crystallography studies (Oh et al., 1994; Oh et al., 1993). This initial comparison was suggested by Nakanishi et al., (1990) for AMPA receptors after examination of the N-terminal regions and the region between M3 and M4.

Chimeras produced between GluR3 and GluR6 subunits by exchanging regions which show homology with LAOBP identified two areas N-terminal of M1 and between M3 and M4 which control agonist selectivity in AMPA and kainate receptors (Stern Bach et al., 1994). These regions were termed S1 and S2 respectively. Exchange of both S1 and S2 regions from the GluR3 subunit to the GluR6 subunit was required to convert the pharmacological profile of the recipient subunit into that of the donor. However the biophysical profile of the recipient was not completely endowed upon the donor. Agonist selectivity was determined by both N-terminal halves of the S1 and S2 regions. The N-terminal half of the S2 domain was shown to be a critical determinant of agonist binding between AMPA and kainate receptors. This has been highlighted by the identification of residues controlling AMPA selectivity in the S2 region. The asparagine at position 721 in the GluR6 subunit has been shown to have a role in controlling agonist selectivity between GluR5 and GluR6 homomeric receptors (Swanson et al., 1997). Thus, the S2 region determines differential low affinity AMPA sensitivity between GluR5 and GluR6 receptors.

The N-terminal half of the S1 was shown to be more dominant for specifying the pharmacological profile of receptor subunits. The effects of swapping S1 and S2 regions were also shown to happen with chimeras of GluR2 and GluR6 subunits (Tygesen et al., 1995).
Studies have also examined the ability of the S1 and S2 regions to bind agonists when expressed alone. The S1 and S2 regions from the GluR4 subunit have been fused together and expressed in insect cells. This fusion protein was shown to have binding properties similar to AMPA receptors suggesting that the S1 and S2 regions are sufficient to form a selective agonist binding site (Kuusinen et al., 1995). This has also been repeated with S1 and S2 domains of the GluR6 subunit with respect to the binding of kainate (Keinanen et al., 1998). The S1 and S2 regions are thought to form two lobes which form a ligand binding pocket analogous to those from the LAOBP (Stern Bach et al., 1994). It has been suggested that the lobes act like a 'Venus fly-trap' catching the ligand between them (Mano et al., 1996).

Homology modelling and site-directed mutagenesis was used to analyse kainate binding properties of chick cerebellar and frog kainate binding proteins and also showed the involvement of S1 and S2 regions (Paas et al., 1996; Wo et al., 1999). A study has reported a high resolution crystal structure of the S1 and S2 regions from the GluR2 subunit in complex with kainate and revealed that both regions formed a bilobar structure similar to the structure of the binding pocket of bacterial periplasmic binding proteins. The study also structurally identified (for the first time) the residues involved in ligand binding for a glutamate receptor (Armstrong et al., 1998). Thus, most studies in this area support the view that the S1 and S2 regions are essential for agonist binding among the ionotropic glutamate receptor family.

1.4.2. Identification of amino acid residues controlling glycine potency in NMDA receptors.

Amino acid residues controlling glycine potency were identified by looking at the S1 and S2 regions on the NR1 subunit. NR1 residues were targeted by analogy to residues controlling agonist potency in non-NMDA receptors and ligand-binding residues of bacterial proteins. Several residues were found that caused large reductions in glycine potency (up to 4 orders of magnitude). Residues were first
located in the S1 region (Kuryatov et al., 1994) at positions Q387 (analogous to the E387 position in non-NMDA receptors) and two amino acid residues further downstream at K465 and F466. However, only mutation of the F466 residue caused an increase in the glycine site antagonist 7-CK IC_{50} by about 200-fold. However, in these mutants the glutamate potency was only decreased by about 10-20 fold compared to wild-type NR1/NR2B receptors. Mutations of amino acid residues that diverge between the N-terminal domains of the glycine-binding NR1 and the non-glycine-binding non-NMDA subunits were shown to give glutamate and glycine EC_{50} values similar to wild-type.

Several other groups identified other NR1 residues in the S1 and S2 regions that control glycine potency of NMDA receptors. Mutations of residues at positions identical to the D463 and K465 residues (in the rat) in the human NR1 subunit caused 100-fold decreases in glycine potency in human NR1/NR2A receptors. This agreed with the findings by Kuryatov et al., (1994). The effect of the mutants were found to be additive because when both mutants were expressed in the same NR1 subunit the glycine potency was reduced by 1000-fold (Wafford et al., 1995). Only the D463A mutation caused a 40-50 fold reduction in the affinity of the glycine-site antagonists, L-687,414 and L-689,560 (Wafford et al., 1995). This contrasts with the observation that the D463K mutation in rat NR1 subunits caused little change in the mutant NR1/NR2B channel IC_{50} for 7-CK (Kuryatov et al., 1994). But it is possible that the change in charge could have produced a different effect or that the antagonists interacted with different residues in the binding pocket.

An S2 mutation at position D732 reduced glycine potency by about 4000-fold but produced little change in 7-CK IC_{50} or other receptor mechanisms such as voltage-dependent Mg^{2+} block or reversal potential (Williams et al., 1996). It was suggested that this residue may not directly bind to glycine in the binding pocket but only couple agonist binding to channel opening. Further S2 mutations at positions F735 and F736 caused 100-fold increases in glycine EC_{50} suggesting that the cytoplasmic region
Figure 1.5. Locations of amino acid residues known to cause large changes in glycine potency (arrows) on the NR1 subunit. Amino acid residues are numbered according to Kuryatov et al., (1994) and Hirai et al., (1996). * Numbered 714 by Wood et al., (1997). ** Numbered 732 by Williams et al., (1996).
between M3 and M4 is also important for glycine potency (Hirai et al., 1996). It should be noted that some of these residues may interact directly with glycine in the binding pocket but some may only couple the agonist binding mechanism with the gating of the channel and not directly bind glycine.

A report has revealed a residue (A714 on the NR1 subunit) not predicted by sequence homology with bacterial binding proteins which caused a 100-fold decrease in glycine potency compared to wildtype (Wood et al., 1997). Insertion of a PKA consensus sequence near this residue (replacement of 4 amino acids) produced a receptor with a glycine potency reduced by 990 fold. Further work has suggested that this residue may be involved in the transduction of the ligand binding signal rather than directly binding glycine (Wood et al., 1999).

The idea that amino acid residues on the NR1 subunit can bind glycine is further supported by the findings from glycine-site antagonist binding studies of cell lines expressing only the NR1 subunit (Grimwood et al., 1995; Ishmael et al., 1996; Lynch et al., 1994). Fusion proteins consisting of only NR1 S1 and S2 regions have been expressed and shown to bind glycine site antagonists (Ivanovic et al., 1998; Sandhu et al., 1999; Uchino et al., 1997). Thus, the NR1 subunit alone may be sufficient to form a glycine-binding site. Moreover, it was also shown that if the MI-III domains were removed and replaced by a peptide linker, antagonist binding was greatly reduced compared to the full length NR1 subunit. Therefore, although the NR1 subunit alone may be sufficient to form a glycine-binding site (Sandhu et al., 1999), additional structural elements within the NR1 polypeptide may be required for efficient formation of the glycine-binding pocket.

1.4.3. Summary-Possible location of residues controlling glutamate potency on NMDA receptors.

Although extensive site-directed mutagenesis studies have revealed the amino acid residues that control glycine potency on the NR1 subunit in NMDA receptors.
Identification of those residues involved in determining glutamate potency has (as of 1996-1997) so far remained elusive.

A possible location is on the NR2 subunit rather than the NR1 subunits of NMDA receptors. This hypothesis is supported by the following evidence:

1. Binding studies show that glutamate binding antagonists bind with high affinity to the NR1 + NR2 expressing cell lines but not NR1 only expressing cell lines. (Grimwood et al., 1996; Lynch et al., 1994).

2. NR2 subunits alone can bind (with low affinity) glutamate site antagonists (Kendrick et al., 1996) (This was published after the project had started).

3. NMDA receptor channels are likely to be heteromeric. It is unlikely that NR1 subunits form functional ion channels alone. (i.e lack of cell death in NR1 only cell lines). Therefore the glutamate site is unlikely to be solely on the NR1 subunit.

4. Amino acid residues on the NR1 subunit at analogous positions to those that cause changes in glutamate potency in non-NMDA receptors and those that are involved in binding in bacterial periplasmic binding proteins, when mutated cause large changes in glycine potency but not in glutamate potency.

1.4.4. Aims.

The aim of this project is to show that residues on the NR2 subunits control glutamate potency, to identify the location of these residues on the NR2A subunit and to show if possible that mutagenesis of these residues results in a disruption of the binding mechanism of the receptor rather than the gating of the channel. This is based on the following assumptions.

1. Residues controlling glutamate potency exist on the NR2 subunit rather than on the NR1 subunit of the heteromeric NMDA receptor.
2. These residues exist at similar positions to that of residues on the NR1 subunit known to cause changes in glycine potency in NMDA receptors.

Residues will be targeted that exist in all NR2 subunits and at similar positions to that of residues on the NR1 subunit known to cause changes in glycine potency in NMDA receptors. Residues will be mutated by site-directed mutagenesis on the NR2A subunit and inward currents from recombinant NR1/mutant NR2A NMDA receptors will be measured from *Xenopus* oocytes expressing the injected subunit mRNA. Agonist dose-response curves will be measured to identify if any of the mutations will cause a reduction in agonist potency compared to wild-type NR1/NR2A receptors. Some mutations will also be generated in the NR2D subunit, to examine how some of these mutations effect glutamate potency in NR1/NR2D receptors and how they compare to their NR1/NR2A counterparts (i.e. are the effects conserved between NR2 subunits?). Additionally glutamate site antagonist affinity will be measured to see if there is any disruption of the antagonist binding site in any of the NR2A mutants which cause a reduction in glutamate potency. The information generated from these experiments will be used to determine whether the glutamate binding site has been disrupted in these NR1/mutant NR2A receptors.

Furthermore, based upon the strategy described by Laube *et al.*, (1998) one of the NR2A mutants will be coinjected with NR2A wildtype cRNA as an attempt to identify the number of NR2A subunits within the NR1/NR2A receptor complex.
1.5. Inducible control of gene expression in the brain using the tetracycline system.

1.5.1. The inducible tetracycline (tet) gene expression system.

The tetracycline inducible gene expression system is based upon the properties of the tetracycline repressor protein (tetR) in *E.coli* and its binding site, the transposon-10 (Tn-10) tetracycline resistance operator. The repressor is expressed widely in gram-negative aerobic bacteria to give resistance against tetracycline (Hillen and Wissmann, 1989). It is involved in repressing the expression of a membrane associated protein which removes tetracycline from the cell. The repressor binds to specific operator sites placed to hinder transcription initiation in the promoter/enhancer region of the membrane associated protein. In the presence of tetracycline, tetR-operator binding is inhibited and repression removed.

The inducible expression system (Gossen and Bujard, 1992) consists of two components one containing a tetracycline transactivator protein (tTA) driven by a cytomegalovirus promoter (driver component) and the other being a transactivator binding minimal promoter (tet-promoter) controlling expression of the gene of interest (effector component). Tetracycline inhibits binding of the transactivator to the minimal promoter preventing gene expression. Later a reverse transactivator (rtTA) was developed by mutating various amino acid residues in the original tTA protein. This produced a transactivator that binds to the tet-promoter in the presence of tetracycline and initiates transcription (Gossen et al., 1995).

The tTA protein is a fusion of binding sites for the tet-operator site from the tet-repressor protein (tetR) and the acidic VP16 activating domain from the herpes simplex virus. In the presence of tetracycline, binding of tTA to the tet-operator site is disrupted and expression is stopped. The tTA binding promoter consists of seven tet-operator sites in tandem fused upstream from a minimal human cytomegalovirus immediate early gene promoter (CMV IE). It was found that a promoter consisting of seven tet-
operators gave high expression levels upon induction but little activity in the presence of tetracycline (Gossen and Bujard, 1992). Positioning of the tet-promoter upstream of the gene of interest allows inducible control by tetracycline. Thus, the promoter has been designed so that its activity or 'leakiness' is minimal in the absence of transactivator protein binding.

Under the control of tetracycline this system can regulate gene expression levels by five orders of magnitude within 24 hours in eukaryotic cell lines (Gossen and Bujard, 1992). The concentration of tetracycline needed is low (~1 µg/ml, although repression by several orders of magnitude was observed at concentrations of 0.1 µg/ml) (Gossen et al., 1993). The uptake of tetracycline into the cell is rapid and additionally there are several chemical analogues available which have the same inducer properties as tetracycline but have a reduced antibiotic activity such as anhydrotetracycline and doxycycline (Gossen and Bujard, 1993; Gossen et al., 1995). The system has also been found to be functional in transgenic mice giving induction ratios of up to several-thousand fold in certain tissue types (Furth et al., 1994). Administration of tetracycline by slow release pellets inserted subcutaneously was found to be adequate to repress expression in transgenic animals. The tet-system is useful because a tissue specific promoter can be placed upstream of the tTA gene to drive expression of a controlled gene in particular cell types. This has been done in the cardiac tissue (Passman and Fishman, 1994) of transgenic mice using the rat α myosin promoter and in pancreatic β-cells using the rat insulin promoter (Efrat et al., 1995).

A study of the kinetics of the tTA and rtTA systems has been done in transgenic mice showing that both systems can used to work effectively in vivo (Kistner et al., 1996). The tTA gene was driven by the liver specific LAP promoter while rtTA was driven by the human CMV IE promoter. The transactivators were used to control the expression of the luciferase gene in mice transgenic for both genes. For tTA/indicator mice, tight control of expression could be achieved in various tissues with a regulation spanning five orders of magnitude. Using 2 mg/ ml of doxycycline in the drinking water suppression was complete within 1 week after administration. However, for the
tTA mice, induction ratios were found to be only 10 fold in the brain which was much lower than that found in other tissues. A similar regulation could be obtained using the induction of expression by rtTA in the presence of doxycycline in certain tissue types. After administration of doxycycline significant induction could be reached after 4 hours and was near to full levels after 24 hours. More importantly background levels of uninduced luciferase expression in rtTA/indicator mice were found to be similar to those found in indicator only mice.

1.5.2. Other inducible gene expression systems.

There are several other systems available to give inducible gene expression. Eukaryotic derived systems include steroid hormone receptor systems and metallothionein promoter based systems. These systems suffer from pleiotropic effects brought on by the inducer (e.g. steroid hormones and heavy metal ions) making the elucidation of a physiological role of an inducible gene particularly difficult. Additionally the controllable promoters in these systems give basal levels of activity ('leakiness') in the uninduced state that are too high (Yarranton, 1992). However use of the dexamethosone-inducible MMTV promoter system in mammalian cell lines has been found to have a low basal activity (in the uninduced state) for the stable expression of receptor subunits (Grimwood et al., 1996; Whiting et al., 1991). How this system would work in vivo, remains to be reported. Moreover, the insect-hormone derived ecdysone-inducible expression system reported induction ratios of 4 orders of magnitude in stable mammalian cell lines (No et al., 1996). A direct comparison of the ecdysone-inducible system showed lower basal activity than the inducible tetracycline system in cell culture.

Prokaryotic based inducible expression systems do not suffer from the pleiotropic effects seen in eukaryotic based systems because of the lack of proteins in mammalian cells which would recognise prokaryotic regulatory elements (Gossen et al, 1993). Other prokaryotic repressor systems exist such as the E.coli derived lac
repressor-operator (LAP267) system which has been designed in the same way as the tet-system (Baim et al., 1991). It is based upon the principle of inducer activation rather than repression of gene expression. The inducer, isopropyl-β-D-thiogalactoside (IPTG) binds to the chimeric transactivator increasing its affinity for the operator and thus allowing transcription to proceed (resulting in faster onset kinetics). This has been a drawback with the tTA system, as expression onset will be limited by the removal of tetracycline from the cell and repression over long periods of time would have to be maintained by applications of tetracycline over long periods of time. This may be undesirable in situations involving transgenic animals or gene therapy. Although the lac repressor-operator system works in tissue culture, induction ratios are only about 1000 fold and the system is temperature sensitive. Additionally, the concentrations of inducer needed to generate an effect approaches levels toxic to cells in tissue culture (Gossen et al., 1993).

The Cre-lox recombination system from bacteriophage P1 uses Cre, a site-specific recombinase to catalyse DNA recombination between two loxP recognition sites (Chambers, 1994). Therefore this can be used to excise genes flanked by two loxP sites. Insertion of loxP sites flanking the gene of interest (by homologous recombination) would allow the inhibition of gene function only when Cre was expressed. As long as Cre is driven by a time and tissue-specific promoter, gene deletion can be restricted regionally. This has been used successfully in transgenic mice to restrict the deletion of DNA polymerase β in T-cells (Gu et al., 1994). Tsien et al., (1996) used a tissue-specific Cre (driven by the CaMkinaseII promoter) to excise a floxed NR1 gene in only CA1 hippocampal cells. This has been the best example of the Cre-lox system in the brain. However the system was not inducible, so temporal control of gene expression would be dependent upon a time-regulated promoter to drive expression of the Cre recombinase.

Various inducible Cre recombinase systems have been developed in mice. The Mx1 interferon responsive promoter has been used to drive the expression of Cre recombinase (Kuhn et al., 1995). Unfortunately, induced deletion of a floxed DNA
**Figure 1.6.** The two component inducible tetracycline system. The driver component drives the expression of the tTA gene. A. In the absence of tetracycline the tTA protein binds to the tet-minimal promoter activating (+) the expression of the gene of interest. B. Addition of tetracycline displaces the tTA from the tet-promoter inhibiting (−) expression of the effector gene. From Mayford et al., (1995).
polymerase B gene after the injection of an interferon analogue was variable in different tissues (only 8 % deletion in the brain). A fusion protein of the Cre protein and a mutated estrogen-binding domain has been created which allowed the ligand-dependent Cre recombinase to be induced by anti-estrogen drugs (tamoxifen) but not by endogenous hormones (Zhang et al., 1996). By using a tissue-specific promoter, Cre recombinase-estrogen receptor fusion protein expression could be restricted to B lymphocytes and induced by tamoxifen (Schwenk et al., 1998). A fusion protein of Cre and the hormone binding domain of a mutated human progesterone receptor has been created which binds to the progesterone antagonist, RU-486 but not progesterone (Kellendonk et al., 1996). This system has been shown to function efficiently in the brain if the expression of the fusion protein is driven by the CaMKIIα promoter (Kellendonk et al., 1999). Such inducible Cre systems would overcome reliance on time-dependent promoters since activators can be given when excision of the gene is required. A form of temporal control of Cre expression has been attempted by driving Cre with the tet-promoter so that Cre expression is controlled by tTA (St-Onge et al., 1996).

Although the Cre recombinase system seems a promising way to completely inhibit gene function via an on/off action, the tet-system has the advantage of reversibility and graded action.

1.5.3. Studies of the inducible tetracycline system expressed in the brain.

Several studies have focused on the use of the tetracycline system in the brain. Most successful efforts have used a simple approach to drive tTA transcription. This has been based on a two component approach where the tTA/rtTA has been driven by a specific promoter in one strain of mice, while another strain contains an 'indicator' or 'effector'.
Mayford et al., (1996) used the CaMkinase II promoter. This promoter was known to drive specific expression in the forebrain. ‘Driver’ mice were crossed with 'effector' mice which contained a mutant form of the CaMkinase II gene. Three lines of double transgenic mice were shown to have specific expression of the mutant CaMkinase II mRNA in the forebrain, hippocampus (dentate and CA1) and in one line expression was only found in the CA1 region of the hippocampus (Mayford et al., 1996).

Expression of the mutant CaM kinase II could be suppressed by 1 mg/ml doxycycline (an analogue of tetracycline) given to the animals in the drinking water for 3-5 weeks and activity of CaMkinase II could be returned to initial levels after 6 weeks withdrawal from doxycycline treated water. However, induction ratios were only 10-fold but this change was great enough to produce a physiological effect by disrupting spatial learning.

A second study used the NSE (neuronal specific enolase) promoter to drive tTA expression (Chen et al., 1998). Different transgenic lines were found to have expression patterns that differed regionally but could achieve induction levels within the brain of up to 5 orders of magnitude. For instance, different lines showed high expression in the striatum, cerebellum or cerebral cortex when crossed with luciferase indicator lines. These mice also contained an autoregulatory tTA cassette where an additional tTA gene is under the control of a tet-promoter (Shockett et al., 1995). Thus, this system utilises a trigenic rather than a traditional bigenic approach. Low doses of doxycycline (25 μg/ml) in the drinking water given for three weeks were shown to be effective in turning off targeted gene expression. Also it was found that maintainence of trigenic mice on lower doses of doxycycline (50 μg/ml) allowed a rapid induction of targeted gene expression and a return to pre-doxycycline gene expression levels once doxycycline had been removed (4 weeks). Higher doses of doxycycline (2 mg/ml) only allowed luciferase to be expressed to 10-15 % of maximal levels before treatment after 8 weeks of doxycycline washout.
In certain cases a rapid induction of gene expression is needed and this has been accomplished by using the tetracycline dependent activator of gene transcription, rtTA. The rtTA was driven by the CaMkinase II promoter and was able to induce full β-galactosidase expression after 6 days of 6-12 mg/ml doxycycline treatment given to double transgenic mice as food. This expression was maintained for as long as the doxycycline was administered and could be hardly detected 3 days after doxycycline withdrawal (Mansuy et al., 1998). However, induction ratios of the effector gene (calcineurin) were only about 2-fold after 2 weeks treatment on 6 mg/ml doxycycline. Basal levels of calcineurin were returned to normal after 2 weeks withdrawal from the drug. A more effective use of this system may be obtained by using more efficient methods of drug delivery to the brain and more potent tetracycline derivatives.

1.6. The use of 'reverse genetics' to study brain function-e.g. learning and memory.

Traditional methods to control gene expression rely upon using pharmacological agonists and antagonists on the target protein. However, drugs only exist for a small set of proteins which are usually not region specific.

The recent application of 'reverse genetics' (mutating a gene to identify the phenotype) has created a number of gene knockout mice allowing specific ablation of protein function. A number of genes thought to be involved in learning and memory have been studied in this way. These have included the tyrosine kinase, fyn (Grant et al., 1992) the serine kinase CaMkinaseIIα (Silva et al., 1992a; Silva et al., 1992b) and various subunits of neurotransmitter receptors such as the NMDA-ε1 subunit (NR2A) of the NMDA receptor (Sakimura et al., 1995). Transgenic mice with these mutations have produced impaired spatial learning and hippocampal LTP. Although the results suggest that these genes are important for LTP itself, linking the process of LTP directly with learning and memory cannot be made (Grant and Silva, 1994; Mayford et al., 1995).
Firstly many genes thought to be involved in the molecular aspects of learning and memory are also important for normal animal development. In some cases producing gross anatomical changes in the brain and complicating the results obtained after behavioural analysis of the animal (e.g. in Fyn mutants there are changes in anatomy in the hippocampus compared to wildtype). Thus, the absence of a gene can produce more than one phenotypic effect in an animal and whether impairment of the animal's performance in behavioural tests and electrophysiological analysis of brain slices is directly due to the role of the gene in LTP or some deficiency in normal development due to its absence cannot be distinguished. Using traditional knockout strategies the function of the knocked out gene must be deduced from the phenotype of animals which have had the gene in question absent throughout development. This could result in compensation by other genes to give little or no phenotype (Gingrich and Roder, 1998). Secondly, because the mutation is not region-specific the knockout is present throughout the entire brain, so even if attention is focused on the hippocampus, another process in another region of the brain e.g. spatial awareness or walking may have been disrupted preventing the animal from performing normally in behavioural tests. Another problem is that when certain genes are knocked out the animal cannot survive without its expression after birth (e.g. NMDAR1 subunit) (Forrest et al., 1994; Li et al., 1994) so that the loss of gene expression cannot be studied in adulthood.

These problems have been partially solved by the use of inducible CaMkinase II mutants under the control of the tetracycline system (Mansuy et al., 1998; Mayford et al., 1996) and the use of the subregional NR1 knockout in hippocampal CA1 cells using the Cre-lox system (Tsien et al., 1996a; Tsien et al., 1996b).

1.7. Summary.

Gene knockouts in mice have provided a high degree of molecular specificity for elucidating the functional role of genes in the brain. But they lack temporal control
and reversible and graded regulation which may be important variables to control (Mayford et al., 1995). Reversibility of the tet-system would allow an inducible gene to express naturally during development until adulthood while termination of expression can be induced when required. Using a tissue-/cell-specific promoter to drive expression of the tTA protein, any disruption of gene function could be restricted to the brain or other tissue-specific cells. Generation of such 'driver' mice would be valuable in the study of the regional-specific effects of proteins in the brain.

1.8. Aims.

I plan to generate a tissue-specific tTA driver mouse based upon the two component system in transgenic mice as developed by Furth et al., (1994). I plan to use a fragment of the mouse NR1 5' upstream region to drive tTA expression. As mentioned earlier the NR1 gene is ubiquitously expressed in the brain and during early stages of development. This would be useful especially in analysing the control of NMDA subunit expression in the brain. This will require the development of a driver to drive expression of the tTA gene under the control of a tissue specific promoter such as the mouse NR1 promoter. To effectively use the driver, the line will have to be mated with an effector line which would allow inducible expression of the tTA controlled gene.

I plan to assemble the construct and generate transgenic mice, containing the driver construct. Since the construct would not need to be integrated in the loci of a specific part of the mouse genome, the process of creating transgenic mice by gene targeting is not required. Thus, transgenic mice can be created by pronuclear microinjection of the DNA construct into single cell mouse eggs. This is a widely used technique to generate transgenic mice where the goal is to gain expression of novel genetic material. Injected DNA integrates into the mouse genome by random integration. This avoids the need to do laborious tissue-culture work involved in gene-targeting by homologous recombination in mouse embryonic stem cell lines. Once this
transgenic line is established any lines containing tet-promoter controlled effector genes can be crossed with these mice to control gene expression.

Transgenic founder mice for the driver constructs will be generated and once germ line transmission of the transgene is established the resulting lines of mice will be crossed to generate double transgenic mice for both driver and indicator. From these double transgenic mice the evaluation of the system will be done.

The effectiveness of tTA expression under the control of the NR1 promoter in the driver mice will have to be characterised in the whole brain (and specific regions of the brain). This will be done by crossing the driver with an indicator mouse which contains reporter genes controlled by the tet-promoter. This will allow the evaluation of the tet-system in the brain under the control of the NR1 promoter. It is important to find out how well the native NR1 promoter will drive the expression of the transactivator and whether the transactivator will mimic the spatial and temporal expression of the NR1 subunit.

2.1. Overview.

Molecular biological methods were based upon protocols from (Sambrook et al., 1989). Data analysis for agonist dose-response curves and Schild plots were performed using the curve fitting program CVFIT (D. Colquhoun and I. Vais, University College London, 1997). Transgenic techniques were based upon protocols from (Hogan et al., 1994).

2.2. Solutions and materials.

2.2.1. NMDA subunit cDNA and mouse NR1 genomic DNA.

The wild-type NR1 and NR2A NMDA cDNA clones were obtained from Dr. Ralf Schoepfer (Monyer et al., 1992). These clones were previously cloned into vectors optimised for RNA expression in Xenopus oocytes (pRSSP series of vectors-(Kuner and Schoepfer, 1996)). Transcription of RNA was driven in vitro by SP6 polymerase. Thus NR1 and NR2A vectors were named NR1-SP-ex1 and NR2A-SP respectively. Mouse genomic NR1 DNA was obtained from Matthias Kneussel, UCL.

2.2.2. General chemicals.

Chemicals used were obtained from BDH-Merck, Fluka biochemicals, Gibco-BRL, Sigma and Tocris.
2.2.3. **Restriction enzymes.**

All restriction enzymes were obtained from either Boehringer Mannheim or New England Biolabs. Enzymes were used with buffers provided according to the manufacturers instructions.

2.2.4. **Bacterial strain.**

For transformations the DH5α *E.coli* strain from Gibco-BRL was used with the following genotype:

supE 44 Δlac U169 (ϕ80lacZΔM15) hsd R17 recA1 endA1 gyr A96 thi-1 relA1

2.2.5. **Bacterial medium and preparation of electrocomponent cells.**

TB (Terrific Broth) medium- 11.8 g SELECT peptone 140, 23.6 g Yeast extract, 9.4 g Dipotassium hydrogen phosphate, 2.2 g Potassium dihydrogen phosphate (Gibco-BRL, 47 g/L)

LB (Lennox L Broth) medium- 10 g bactotryptone, 5 g bactoyeast extract, 5 g NaCl (Sigma, 25 g/L)

LB Agar -(Gibco-BRL, 32 g/L).

Transformations were done using frozen bacterial stocks, which were prepared according to the technique described by (Dower *et al.*, 1988). Bacteria were streaked (from a frozen glycerol stock kept at -80 °C) on to an LB-agar plate and incubated overnight at 37 °C. A single, well isolated colony was grown overnight as a small scale culture at 37 °C. This was used to start a large-scale culture using 500 ml LB-
medium. Cells were grown up until the cell density reached an \( \text{OD}_{600} = 0.5-1.0 \). Cells were then centrifuged at +4 °C for 15 minutes at 4,000g and the medium removed. The pellet was resuspended carefully in 500 ml ice-cold Milli-Q water and centrifuged again at 4,000g. This process was repeated twice, again in ice-cold water and then in 20 ml 10 % glycerol. Finally the cell pellet was resuspended in 10 % glycerol in a 1:1 (cells:10 % glycerol) ratio. The cells were aliquotted and frozen in liquid nitrogen before being stored at -80 °C. Electrocompetence was tested by transforming 10 pg SKII- Bluescript circular vector (Stratagene), using the protocol described later. Cells usually gave 1-2 x 10\(^7\) colonies/µg DNA on an LB-agar plate supplemented with 200 µg/ml ampicillin.

2.3. Methods-Subcloning.

2.3.1. General subcloning: Digestion reactions

A typical analytical digestion reaction was made of the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x restriction enzyme buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Miniprep/maxiprep DNA</td>
<td>1.0 µl (approx. 0.5 µg)</td>
</tr>
<tr>
<td>(10xBovine Serum Albumin)</td>
<td>1.0 µl if required</td>
</tr>
<tr>
<td>Restriction enzyme</td>
<td>0.5 µl (approx. 5 U)</td>
</tr>
<tr>
<td>(2nd restriction enzyme)</td>
<td>0.5 µl if required</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>made up to a final volume of 10 µl</td>
</tr>
</tbody>
</table>

Preparative digestion reactions of DNA contained 1x restriction enzyme buffer (NewEnglandBiolabs/BoehringerMannheim), 1-5 µl mini/maxi-prep DNA backbone/insert, 0.5 µl restriction enzyme at a concentration of 5 U/µl (New England biolabs/Boehringer Mannheim) and Milli-Q water (Millipore) making the final volume
10 μl. Reactions were carried out usually at 37 °C for 1 hour unless otherwise specified.

In certain cases, where restriction enzymes worked in incompatible buffers. Digestions were carried out in separate reactions (with the low-salt buffer preferring enzyme used first and the reaction supplemented with more salt for the second enzyme reaction), or if the buffers were of completely different compositions, separately with the DNA purified and desalted before using the second enzyme in the optimal reaction buffer.

For preparative digestions, fragments were separated by gel electrophoresis in a 0.8% SeaKem GTG agarose (Flowgen) gel made in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)). This was done in electrophoresis tanks (Flowgen) filled with 1x TAE buffer.

Voltage was provided by a Power pac 300 power supply (Biorad) at a speed of 2.5-5 V/cm. To estimate the molecular size of the DNA bands 1.0 μg λ-bacteriophage (Pharmacia Biotech) digested with Sty I (Figure 2.1), was added in an adjacent well. To visualise DNA under UV light (wavelength = 302 nm) 3 μl 10 mg/ml ethidium bromide was added to 100 ml liquid agarose. The DNA band of the appropriate size was excised from the gel and extracted using the JETSORB© gel extraction kit from Genomed (ams biotechnology Ltd). The DNA fragment isolated, was resuspended in 20 μl 1x TE buffer for ligation reactions.

For analytical digestions, DNA was analysed by gel electrophoresis in a 0.8% SeaKem LE agarose gel (Flowgen) made in 1x TAE buffer. 10 % Ficoll loading buffer was added to all DNA reactions prior to loading (15 % Ficoll, 0.25 % Xylen cyanol FF, 0.25 % Bromophenol blue dissolved in water).
Figure 2.1. Gel photo of DNA markers. Panel A shows the DNA marker λ/Sty I run in an 0.8 % agarose gel in 1x TAE buffer. Panel B shows the KS+/Msp I marker run in an 8 % polyacrylamide gel in 1x TBE buffer.
2.3.2. Polymerase Chain Reaction (PCR)-subcloning.

Site-directed amino acid residue exchanges were introduced into wild-type NR2A subunits using PCR based strategies. Reactions were carried out in an UNO-Thermoblock® thermocycler (Biometra) under the following conditions:

- Initial denaturation: 94 °C for 2 minutes
- Denaturation: 94 °C for 30 seconds
- Annealing: 40-55 °C for 15-30 seconds
- Polymerase extension: 72 °C for 1.5-2 minutes
- Repeat denaturation, annealing and extension cycle x19

- Final extension: 72 °C for 5 minutes

Some optimisation was performed to improve the yield of PCR products by altering the temperature and the duration of some of the cycles.

PCR mixture (total volume 50 μl):

- 1 μl 1:10 (~100 ng)/1:100 (~10 ng) dilution of DNA template (wild-type NR2A) in water.
- 1 μl sense primer (10 μM).
- 1 μl antisense primer (10 μM).
- 2 μl 5 mM nucleotide mix.
- 5 μl 10 xTaq polymerase (Promega) / Taq extender buffer (Stratagene-if required).
- 37.5 μl Milli-Q water.
- 0.5 μl Taq polymerase, 5U/μl (Promega).
Negative PCR controls were carried out in the absence of template DNA.

After completion, reactions were purified with 1x volume of phenol:chloroform:isoamylalcohol mix (Gibco-BRL) in a ratio of 25:24:1 and precipitated with 1/10 volume 3 M NaAc pH 5.2 and 2.5 volumes of ice cold 100% ethanol. Mixtures were spun in a microfuge at +4 °C at 15, 800g for 20 minutes. The DNA pellet was desalted with 350 μl 70% ethanol spun in a microfuge for 5 minutes and then washed again with 50 μl 70% ethanol.

The pellet was allowed to air-dry and resuspended in the appropriate restriction enzyme digestion buffer. Reactions were carried out as mentioned before.

Smaller PCR DNA fragments (<400bp) were analysed on 8% polyacrylamide gels (For 5 ml gel: 1 ml 40% acrylamide solution (39:1 acrylamide:bisacrylamide), 4 ml 1x TBE buffer, 50 μl 10% APS, 5 μl TEMED) made in 1x TBE buffer (10 x TBE buffer- 45 mM Tris-borate, 1 mM EDTA (pH 8.0) from National diagnostics). Electrophoresis was carried out using the Mini-PROTEAN II electrophoresis cell (Biorad). Gels were run at a speed of 10-15 V/cm until the loading buffer marker had reached 3/4 of the distance down the gel. Gels were stained for 20 minutes in ethidium bromide solution and then destained for a few seconds before visualisation under UV light. Sizes of DNA bands were estimated using 1.0 μg Bluescript KS+ plasmid DNA (Stratagene) digested with Msp I (Figure 2.1). DNA fragments of the correct size were isolated by overnight diffusion from the excised gel slice in 50 μl Milli-Q water and desalted (as described above) before it was used in ligation reactions.

2.3.3. Ligation reactions.

Ligation reactions contained 1x T4 ligase buffer (Boehringer Mannheim), the appropriate concentrations of backbone/insert DNA for efficient ligation, 0.5 μl T4
ligase (5U/μl) (Boehringer Mannheim) and Milli-Q water to make the final volume up to 10 μl. Reactions were left overnight at 14 °C.

2.3.4. Electroporation of bacteria.

1-2 μl of a ligation reaction was added to a 1 mm gap BTX disposable electroporation curvette on ice (BTX Inc.). 40 μl of electrocomponent DH5α *E.coli.* was added to the curvette and mixed on ice. Bacteria were transformed in a BTX electro-cell manipulator®-600 with a resistance of 129 Ohms and a voltage of 1.6 kV. Immediately afterwards, 600 μl of pre-warmed SOC medium (2.0 % w/v bacto-tryptone, 0.5 % w/v bacto-yeast extract, 8.5 mM NaCl, 0.25 mM KCl, 10 mM MgCl₂, 20 mM glucose) was added to the curvette and the cells were allowed to recover for 15-20 minutes at 37 °C in a shaking incubator (250 rpm). Cells were then spread on LB-agar plates supplemented with 150 μg/ml ampicillin (Sigma).

2.3.5 Small scale preparation of plasmid DNA from bacterial cultures (Mini prep).

Small-scale mini-preparation of plasmid DNA was based upon the protocol for alkali lysis of bacteria (Birnboim and Doly, 1979; Ish-Horowitz and Burke, 1981). Single, well isolated ampicillin-resistant colonies appeared after 12-16 hours incubation at 37 °C. Individual colonies were picked using a sterile toothpick and grown as small scale cultures at 37 °C and shaken at 250 rpm. Cells from overnight, small-scale cultures (1.5 ml) were centrifuged (8,000g) in a benchtop centrifuge-5415C (Eppendorf) and the medium aspirated. This was repeated twice to remove as much medium as possible. The cell pellet was resuspended (on ice) in 150 μl solution I (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)) and then lysed with 300 μl of freshly prepared solution II (0.2 N NaOH, 1 % SDS). The mixture was neutralised by 200 μl solution III (3.38 M CH₃-COOK, 5.60 M Glacial acetic acid) and
incubated on ice for 5 minutes before being centrifuged for 5 minutes at 15,800g. The supernatant was kept and contaminant RNA was digested by adding 3 µl 10 mg/ml RNaseA (Sigma) and incubating the mixture for 30 minutes at 37 °C. Protein was removed from the remaining DNA solution by adding 400 µl phenol:chloroform:isoamylalcohol mix (Gibco-BRL) in a ratio of 25:24:1 respectively. The mixture was vortexed for 2 minutes and centrifuged for 2 minutes at 15,800g. The DNA was precipitated by adding 850 µl 100% ethanol. The mixture was mixed and centrifuged at 15,800g. The remaining DNA pellet was desalted with 500 µl 70% ethanol and then resuspended in 50 µl 1 x TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

2.3.6. Confirmation of positive clones.

Clones were confirmed by analytical restriction enzyme digestion and automated DNA sequencing (Dye-Deoxy-Terminator Cycle sequencing kit) using an Applied Biosystems (ABI) 373 sequencer. Sequencing was done using 3 µl mini-prep DNA, this was further purified by adding 2 µl cold 4M NaCl and 10 µl 13 % PEG (BDH-Merck), the total volume of the mix was made up to 20 µl using Milli-Q water. The mix was incubated for 20 minutes on ice and then centrifuged at 15,800g at +4 °C for 20 minutes. The remaining DNA pellet was desalted using 70 % ethanol. The dried pellet was resuspended in 1 µl sequencing primer (10 µM), 11 µl Milli-Q water and 8 µl Dye terminator cycle sequencing ready reaction, with AmpliTaq® DNA polymerase (ABI PRISM, Perkin Elmer). Reactions were carried out under the following PCR conditions:

Initial denaturation: 96 °C for 2 minutes

Denaturation: 96 °C for 30 seconds
Annealing:55 °C for 15 seconds
Polymerase extension: 60 °C for 4 minutes
Repeat denaturation, annealing and extension cycle x 24

Sequencing reactions were precipitated with 3 µl 2 M NaAc (pH 4.5) and 50 µl 100 % ethanol. This mixture was incubated on ice for 10 minutes and then centrifuged for 30 minutes at 15,800 g at +4 °C. The remaining pellet was washed with 50 µl 70 % ethanol. Before loading, the pellet was resuspended in 4 µl sequencing loading dye (5:1 ratio, deionised formamide:50 mM EDTA ), denatured for 2 minutes at 96 °C and then placed on ice.

Clones were sequenced along the entire length of the PCR segment inserted into the DNA construct. Sequencing was carried out in both sense and antisense directions. Sequencing data was analysed with the sequence analysis software, Sequencer™ v3.1 (GeneCodes Corp, MI). Cells containing confirmed NR2A mutant constructs were grown up as a large scale overnight culture (40 ml TB-Amp-medium) and DNA was isolated using the Qiagen Maxi-prep DNA purification kit (Qiagen).

2.4. Insertion of point mutations in the S1 and S2 regions of the NR2A subunit.

2.4.1. Site-directed mutagenesis-

NR2A(N463A), NR2A(K465E), NR2A(H466A) and NR2A(H466F) (Figure 3.3).

These mutations were generated using a common sense primer, rNR2A-PCR21-s(5'-3'):

\[
\begin{align*}
\text{GACGCTGAGCCTGAGGCAtGCTGTG} & \text{CTGCAAGGACATCGTACGCTC} \\
& \text{CTGACTGCGAGCAGATGACAA} \text{CTaAGCATTG}
\end{align*}
\]

BglII SphI

AfIII
This contained the Blp I site for cloning and introduced unique Sph I and Afl II sites by exploiting the redundancy of the genetic code. These sites were used to identify clones containing the point mutation. *NR2A wild-type sequence is in upper case while mutated nucleotides are in lower case. Restriction enzyme sites are underlined and the mutated codon corresponding to the point mutation is in bold.*

This primer was used with the following antisense primers to generate the mutated PCR products. For NR2A(N463A), rNR2A-Mut20-a(5'-3'):

\[
cgcGTTAACCTTTTTCCCATGCTTCCCAgcGGTCACCAG
\]

*HpaI*

For NR2A(K465E), rNR2A-Mut21-a(5'-3'):

\[
cgcGTTAACCTTTTTCCCATGCTcCCCATTGGT
\]

*HpaI*

For NR2A(H466A), rNR2A-Mut22-a(5'-3'):

\[
cgcGTTAACCTTTTTCCCAgcCTTCCCATTGGT
\]

*HpaI*

For NR2A(H466F), rNR2A-Mut32-a(5'-3'):

\[
cgcGTTAACCTTTTTCCCAaaCTTCCCATTGGT
\]

*HpaI*

The DNA template for generating the PCR products (319 bp) was NR2A wild-type cDNA for NR2A(N463A) and NR2A(K465E). PCR products were digested with Blp I and Hpa I for insertion. However, because of the length of rNR2A-PCR21-s obtaining a clean PCR product was difficult. Therefore, for NR2A(H466A) and NR2A(H466F) a shorter primer was used 5' of the Blp I site, rNR2A-Seq3s(5'-3'):

\[
CTTGTGGTGATCGTGCTGAACAAG
\]

The NR2A(N463A) construct was used as the DNA template and the PCR products were cloned into NR2A wild-type cDNA to exploit the use of the inserted diagnostic digestion sites.
2.4.2. Site-directed mutagenesis-NR2A(G669A) (Figure 3.4).

This was generated using the sense primer, rNR2A-Mut23-s(5'-3'):

\[ \text{GACGGTGACCcATAcGACAGAGGAATATTcCGgAACAC} \]

\[ \text{KpnI} \quad \text{BspEI} \]

This was used with the antisense primer, NR2A-PCR20-a(5'-3'):

\[ \text{gcagatcTAGAAGAGGTGCTCCCAATGAAGGT} \]

This primer covered a region which contained a unique Cla I site suitable for cloning.

These primers generated a 484 bp PCR product which when digested with Kpn I and Cla I gave a 388 bp fragment and was cloned into NR2A wild-type cDNA. The unique BspE I site was introduced with the point mutation.

2.4.3. Site-directed mutagenesis-NR2A(T671A) and NR2A(S670A) (Figure 3.4).

The BspE I site introduced with the NR2A(G669A) mutation and the Kpn I site was used to insert these two point mutations. Since the region between the two sites was only about 30 bp long the mutations could be inserted using complementary oligos. Complementary base-paired sense and antisense oligos containing the point mutations and the sticky cohesive ends of Kpn I and BspE I were cloned into the Kpn I-BspE I NR2A(G669A) backbone. For NR2A(S670A) the diagnostic HaeII site was inserted with the mutation.

\[ \text{rNR2A-Mut24-s(5'-3')} \]

\[ \text{CCAATGGeCgctACAGAGGAATAT} \]

\[ \text{HaeII} \]
For the NR2A(T671A) mutation a similar design was used with a diagnostic BamH I site inserted with the mutation.

### 2.4.4. Site-directed mutagenesis-NR2A(V666A) (Figure 3.4).

The Hpa I and BspE I sites were used to insert this mutation. The primer, rNR2A-Seq3-a was used with the antisense mutation primer, rNR2A-Mut27-a(5'-3'):

\[
\text{GTTGT} \text{cCgAATATTCTCTCTGTACTTCCAT} \text{GgGTTg} \text{GT} \text{aCCAAA}
\]

A 616 bp Hpa I-BspE I digested PCR product was generated and inserted into the vector backbone of NR2A(G669A). By introducing the V666A mutation in the NR2A cDNA, the Kpn I site was shifted by 5 nucleotides 5' from the original site. No diagnostic site was inserted and positive clones were identified by sequencing.
2.4.5. Site-directed mutagenesis-NR2A(G664A) and NR2A(T665A) (Figure 3.5).

The NR2A(G664A) construct was generated using the Hpa I-Kpn I sites of the NR2A wild-type vector backbone. Again rNR2A-Seq3-s was used with the antisense primer, rNR2A-mut37-a(5'-3'):

\[
\text{CTTCCATTGGGTACCGTcGcGAAATCGGAAAGGTGGAG}
\]

\[\text{KpnI} \quad \text{NruI}\]

The unique Nru I site was introduced with the mutation.

The NR2A(T665A) mutation was generated in a similar fashion but using a Hpa I-BspE I digestion in the NR2A(G669A) vector backbone. The rNR2A-Seq3-s primer was used with the antisense primer, rNR2A-Mut38-a(5'-3'):

\[
\text{TTGTTCGgATATTCTCTCTGACTTCCATTGGGTACgGegCCAAATC}
\]

\[\text{BspEl} \quad \text{NarI/KasI}\]

The Kpn I site was destroyed and replaced with a Nar I/Kas I site.

2.4.6. Site-directed mutagenesis-NR2D(T692A) and NR2D(K486E) (Figure 3.13).

The NR2D(T692A) construct was generated using the Xba I-BspE I sites of the NR2D wild-type vector backbone. A 916 bp fragment was produced containing the mutation using the primers rNR2D-Seq1-s(5'-3'):

\[
\text{CCCTTCACTGGTGTCATCTCCCT}
\]
and rNR2D-Mut1-a(5'-3'):

\[ \text{TTGCTCCGGATGTTTTTCCG} \text{eGGACCATTG} \]

\[ \text{BspEI} \quad \text{Sac II} \]

The mutation created a new Sac II site which was used for diagnostic purposes.

The NR2D(K486E) mutation was generated using a four-primer strategy. This involved producing two separate PCR fragments (PCR 1 and PCR 2) which created a 70 bp overlap (the mutation was inserted near the BspEI site in PCR 2). A final PCR was carried out using a 1:1 mixture of these fragments as a template and using the two 'outside' primers, so as to produce a single PCR fragment (916 bp) incorporating both PCR 1 and 2 fragments. The fragment was digested with Xba I and BspE I and ligated with the digested vector backbone. The primers for PCR 1 were, rNR2D-Seq1-s and rNR2D-Seq13-a(5'-3'):

\[ \text{GCCGTTGGTAAACCAGG} \text{TAGAGGTCATA} \]

The primers for PCR 2 were, rNR2D-Mut2-s(5'-3'):

\[ \text{TCTACCTGTGTTACCAA} \text{CGGCGAGCATGGCAAG} \text{AAaATCGAT} \]

\[ \text{Cla I} \]

and rNR2D-Seq14-a(5'-3'):

\[ \text{GCTCGTCATAGATG} \text{AAGCGTCC} \]

The consensus dam methylation sequence 'gATC', was removed and changed to, 'aATC' so that Cla I could be used as a diagnostic site.
2.5. *In vitro* cRNA synthesis from wild type/mutant NMDAR cDNA constructs.

RNA was synthesised from maxi-prep/mini-prep DNA using a protocol modified from the RiboMax™ large scale RNA production system using SP6 RNA polymerase (Promega, Madison, WI). All solutions were handed with gloves to avoid RNase contamination and glassware and solutions were washed/made up from diethylpyrocarbonate (DEPC)-treated water. DEPC-treated water was made by adding 0.1 % DEPC (Sigma) to 1 litre Milli-Q water and incubated overnight at room temperature. To inactivate the DEPC, the water was autoclaved before use.

2.5.1. Template linearisation.

All DNA constructs were linearised using a unique Mlu I site inserted after the inserted polyadenylation tail (A NotI site was used for the NR2D constructs). Approximately 10 μg maxi-prep DNA was added to a 100 μl digestion mixture containing 1 x Buffer H (Boehringer Mannheim) and 30U Mlu I (Boehringer Mannheim). The remaining volume was made up with Milli-Q water and the mixture was incubated for 1 hour at 37 °C. For analysis, the digestion was checked by taking a 1.0 μl sample of the reaction and loading this on an analytical agarose gel.

The digested DNA was cleaned by P:C:I extraction (see 2.3.2) followed by a chloroform extraction. The DNA was precipitated with 3M NaAc (pH 5.2) and 100 % ethanol (see 2.3.2) and desalted with 70 % ethanol.

2.5.2. cRNA synthesis.

Assuming that 50 % of the digested DNA had been recovered, the 20 μl RNA synthesis reaction was composed of the following:
8.5 μl DNA template resuspended in DEPC water.

4.0 μl Mix containing 25 mM ATP, UTP, CTP and 8 mM GTP (Promega)

1.5 μl 10 mM m⁷G(5')ppp(5')G, sodium capping

(Pharmacia Biotech, Uppsala, Sweden)

4.0 μl 5 x SP6 polymerase buffer (Promega)

2.0 μl SP6 polymerase mix (Promega)

The reaction was incubated for 37 °C for 1 hour. A 1.0 μl sample of the synthesis was taken for analysis on an DNA agarose gel.

2.5.3. cRNA purification.

The RNA reaction was made up to a total volume of 100 μl with Milli-Q water. To this, 20 μl 2 M NaAc (pH 4.0), 50 μl water saturated phenol (pH 4.0) and 10 μl chloroform was added. The mixture was vortexed and centrifuged as described before. One volume of isopropanol was added to the aqueous phase and incubated for at least one hour at -20 °C. The precipitated RNA was spun down at 15,800g for 5 minutes in a benchtop microfuge. To remove remaining unincorporated nucleotides, the pellet was resuspended in 100 μl water, 30 μl NH₄Ac and 260 μl 100 % ethanol. The mixture was centrifuged and the pellet was washed with 50 μl 70 % ethanol. The remaining pellet was resuspended in 30 μl nuclease-free water (Promega) and stored at -80 °C. A 1.0 μl sample of the RNA synthesis was run on a denaturing RNA agarose gel to check the yield and integrity of the RNA.

2.5.4. RNA gel electrophoresis.

A 60 ml 0.8 % agarose SeaKem GTG (Flowgen) RNA denaturing gel was made with the following components:
5 x MOPS (0.1 M MOPS-BDH-Merck, pH 7.0, 40 mM NaAc, 5 mM EDTA, pH 8.0) buffer 12 ml
40 % formaldehyde (Gibco-BRL) 11 ml
0.48 g agarose SeaKem GTG (Flowgen) DEPC-water 37 ml

The agarose was first dissolved in the water before the 5 x MOPS buffer and the formaldehyde was added. RNA samples were loaded in the following way. Sample buffer (for each 1.0 μl RNA) was composed of 1.5 μl 5 x MOPS buffer, 2.6 μl formaldehyde and 7.5 μl formamide. Loading buffer contained 2.0 μl Ficoll loading buffer and 0.5 μl 1 mg/ml ethidium bromide. Both buffers and the RNA sample were heated to 65 °C for 2 minutes before 11 μl sample buffer and 2.5 μl loading buffer was mixed with the RNA sample. The mixture was loaded on to the gel immediately. The gel was run at a speed of 15/V in 1 x MOPS buffer for 1 hour. A 4 μl sample of RNA marker (1 μg/μl-Promega) was run with the sample to identify the molecular size of the RNA sample.

2.6. Oocyte injection and culture.

Typically NR1 and NR2A wild-type or mutant RNA was mixed in a nominal ratio of 1:1. Mixtures were subsequently diluted with nuclease-free water by 100 to 1000 times (~ 5 ng/μl) to yield adequate expression levels (approximately > 0.5 μA). For NR1/NR2D expression, cRNAs were mixed in a 1:9 ratio respectively and injected undiluted.

Mature *Xenopus laevis* were anaesthetised with 0.2 % tricaine methanesulphonate (pH 5.6) and the ovarian lobes were removed surgically. Oocytes (Stage V-VI) were dissected manually and incubated for 1 hour in 30 μg/μl collagenase (Type V, Sigma) with shaking, at room temperature. Afterwards, the oocytes were washed several times and incubated overnight at 19 °C in modified Barth's solution (in mM): NaCl 88, KCl 1, NaHCO₃ 2.4, Ca(NO₃)₂ 0.33, MgSO₄ 0.82, CaCl₂ 0.44, Tris-
Cl 15, adjusted to pH 7.4 with NaOH, supplemented with 50 IU/ml penicillin (Gibco BRL, Bethesda, MD) and 50 μg/ml streptomycin (Gibco BRL). The following day, oocytes were manually defolliculated and injected using a Drummond Nanoject injector (23 nl cRNA mixture per oocyte). Oocytes were incubated for 2-3 days at +19 °C in modified Barth's solution supplemented with 30 μM D-APV (Tocris). Oocytes were stored at +4 °C until they were used for electrophysiological measurements (up to 7 days after injection). During storage at +4 °C, modified Barth's solution was exchanged every day to prolong oocyte viability.

2.7. Whole oocyte recording in two-electrode voltage clamp configuration.

Responses to glutamate and glycine were measured with a two-electrode voltage-clamp amplifier (TEC05, NPI electronics, Tamm, Germany) at -60 mV using 0.5-2.0 MΩ electrodes filled with 3 M KCl. Electrodes were made from thin wall glass capillaries with filament (1.5 mm O.D. x 1.17 mm I.D.-Clark Electromedical instruments, Pangbourne, U.K.) and pulled using a DMZ-Universal puller (Zeitz-Instruments, Augsburg, Germany).

Glutamate stock solutions (Fluka) (1 M in water) were neutralised with N-methyl-D-glucamine (Fluka). Glycine stock solutions (Fluka) (0.1 M) were made in water. All stock solutions were stored at -20 °C. APV stock solutions (Tocris) (100 mM) were neutralised with NaOH.

Upon penetration with microelectrodes, oocytes were at first perfused in a high Ca²⁺ NFR solution (in mM): NaCl 115, KCl 2.5, HEPES 10, adjusted to pH 7.2 with HCl and supplemented with 1.8 mM CaCl₂. If the oocyte was suitably stable, the perfusion was changed to a nominally Ca²⁺ free, low Ba²⁺ NFR solution (0.18 mM BaCl₂). For glutamate evoked responses, low Ba²⁺ NFR was supplemented with 30 μM glycine. For glycine evoked responses, the NFR was supplemented with 30 μM -10 mM glutamate, depending on the saturating glutamate concentration (from the dose-
response curves) for each mutant. Application of solutions was controlled by the LabVIEW based computer program, Eggworks (Kuner and Schoepfer, 1996). For recording, all oocytes were perfused at a rate of 1.5 ml/min.

Data was recorded on a chart recorder and to computer hard disk. For digital recording, data was filtered at 100 Hz, digitized at 300 Hz and then reduced to a final sampling rate of 10 Hz (oversampling with equal weight averaging 30 samples) before storage.

2.7.1. Agonist dose-response curves.

Agonists were applied for as short as possible until the response reached a plateau or maximum. Agonists were applied in increasing concentrations to generate dose-response curves.

Dose-response data (magnitude of response) was fitted for each oocyte with the Hill equation:

\[ I = \frac{I_{\text{max}}}{1 + (EC_{50}/[A])^n_H} \]  
(Eqn. 1)

where $n_H$ is the Hill coefficient, $I_{\text{max}}$ is the maximum current, $[A]$ is the agonist concentration and $EC_{50}$ is the concentration of agonist required to elicit a half-maximal response. Data was fitted using the least squares fit analysis program CVFIT (Colquhoun and Vais, 1998 University College London). Each data point was then normalised to the fitted maximum of the dose-response curve. Normalised values were then pooled for each oocyte and the averaged values were then refitted with the Hill equation.
2.7.2. **Schild analysis of APV and CPP on NR1/NR2A(T671A) receptors.**

APV antagonism was examined by using the Schild method (Arunlakshana and Schild, 1959). Oocytes were perfused in a solution containing 1.8 mM BaCl₂. Oocytes were perfused for at least 60 seconds in APV before the application of glutamate. Partial, low concentration glutamate dose-response curves were measured, firstly in the absence and then the presence of increasing concentrations of APV. Concentrations of glutamate were increased to match the current responses generated in the presence of APV.

The partial dose-response curves from each oocyte were then fitted with the low-concentration limit of the Hill equation. This can be described by the power function:

\[ y = \left( \frac{x}{x_0} \right)^n \]  
(Eqn. 2)

When plotted on a log-log scale this gave a straight line which can be fitted to the data as a set of almost parallel lines. These lines were then constrained to be parallel by fitting the same Hill slope, \( n \) to all the lines. From these parallel lines, dose ratios, \( r \) were measured (where the dose ratio is defined as the ratio of glutamate concentrations in the presence and absence of APV required to give the same response).

Mean dose ratios from several oocyte recordings were used to generate a Schild plot, where \( (r - 1) \) was plotted against [APV] on a log-log scale.

The data was at first fitted with a straight line (using the power function), if this had a slope sufficiently close to unity. The data was refitted with the Schild equation, where for a competitive antagonist the slope of the line is unity at equilibrium:

\[ (r - 1) = \frac{[\text{APV}]}{K_B} \]  
(Eqn. 3)
The intercept of the plot on the x-axis gave the equilibrium constant for antagonist binding, $K_B$.

The same methods were used to analyse the antagonism of R-CPP (Tocris) on NR1/NR2A(T671A) receptors.

2.7.3. Expression of NR1/NR2A/NR2A(T671A) cRNA mixtures in oocytes.

Mixtures of NR1, NR2A and NR2A(T671A) cRNAs were injected into *Xenopus* oocytes as 1:2:1 and 1:1:2 ratios respectively. The ratio of NR1/NR2A cRNAs were kept constant. Glutamate dose-response curves were generated as described in 2.7.1. In order to identify the number of 'intermediate' forms of the NMDA receptor, containing both wild-type and mutant NR2A subunits, dose-response curves were fitted with the 3- or 4-component form of the Hill equation.

2.8. Generation of tTA 'driver' construct- Isolation of 129Sv mouse NR1 5' UT (untranslated) region.

The 5'UT NR1 region was isolated from a λ-phage clone (36.1.1). This λ-phage clone was obtained previously by library screening by Matthias Kneussel-UCL. The library was made from mouse DNA from the 129Sv substrain (Stratagene). The clone contained genomic DNA from exon 1 to an unknown length of 5'UT region. The length of the 5'UT available and mapping was carried out using restriction enzyme digestion and Southern blotting.

2.8.1. Southern blotting and radiolabelled probe hybridisation.

DNA from plasmid or λ-phage was digested (3-4 hours, 37 °C) and then run in a 0.6 % agarose TBE gel at 1.75 V/cm overnight. Gels were then denatured (1.5 M
NaCl, 0.5 M NaOH) for 1 hour (1.5-2 hours for genomic blots) and then neutralised (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA) for 30 minutes with gentle shaking. Gels were rinsed in MilliQ-water to remove excess neutralisation solution. For plasmid/lambda DNA gels, pressure blotting was carried out for approximately 1 hour at 75 mmHg using the Posi-Blot 30-30® apparatus in 0.3x SSPE (20x SSPE stock-3 M NaCl, 200 mM NaH₂PO₄·H₂O, 20 mM EDTA, pH 7.4) and following the instructions provided with the equipment (Stratagene). For genomic blots overnight capillary transfer was used in 10x SSPE (Sambrook et al., 1989).

After blotting, nylon membranes (Hybond-N, Amersham Pharmacia Biotech) were crosslinked in a Stratalinker® UV crosslinker 2400 (exposure = 120,000 μJ) (Stratagene) and then prehybridised (10 ml 2 x hybridisation solution-(12x SSPE, 10x Denhardt’s reagent and 100 μg/ml yeast tRNA), 10 ml formamide and 1 ml 10 % SDS) for 1 hour-overnight at 42 °C. Additionally 150 μl 10 mg/ml fragmented single-stranded salmon sperm DNA (Sigma) was added to further reduce unspecific binding. DNA fragments used for probe making were digested with restriction enzymes and run at 2 V/cm in a low-melting-point 0.7 % agarose gel. The appropriate fragment was excised and water was added according to the ratio of 3 ml water to 1 g gel slice. Radioactive DNA probes were generated using the Primer-It © labelling kit (Stratagene) and incorporated the radioisotope (α³²P)-dATP (Specific activity: 3000 Ci/mmol) (Amersham Pharmacia Biotech). Probes were hybridised to membranes (in a smaller volume of prehybridisation solution) overnight at 42 °C and these were washed the next day in 5x SSPE, 0.1 % SDS for 30 minutes at 65 °C and twice in 0.3x SSPE, 0.1 % SDS for 10 minutes at 65 °C. Blots were exposed 1 hour-overnight/longer to X-ray film (X-OMAT/AR, Kodak) at ~80 °C.

2.9. Large-scale purification of plasmid DNA by CsCl method.

A 100 ml overnight culture was spun down at 4000g for 15 minutes and the supernatant discarded. Solutions I, II and III were the same as those described for the
miniprep procedure. The pellet was resuspended in 30 ml miniprep solution I on ice. Then 60 ml fresh solution II was added and shaken for 5 minutes followed by the addition of 45 ml solution III (shaken for 15 minutes). The mixture was centrifuged for 15 minutes at 11,300g at +4 °C.

The supernatant was removed carefully and to this 125 ml ice cold 100 % ethanol was added. The mixture was again centrifuged for 15 minutes at 11, 300g. The supernatant was discarded and the DNA pellet was resuspended in MilliQ-water to make the final volume 9 ml. The solution was transferred to a 15 ml screw cap tube and 9 g CsCl (Gibco-BRL, optical grade) was added. The tube was inverted until the salt was completely dissolved.

Using a 20 G syringe, 300 μl 10 mg/ml ethidium bromide solution was loaded into two Buckman tubes. The 9 ml DNA/CsCl solution was divided between the two tubes and these were balanced with CsCl:water (1:1) solution. The tubes were heat sealed and then ultracentrifuged in an NVT 90 rotor (Beckmann) at 80,000 rpm (XL-80 ultracentrifuge, Beckmann) overnight at 20 °C.

The following day, the top of the tube was pierced with a syringe needle, and a second needle was used to collect the layer of circular plasmid DNA. The same ultracentrifugation procedure was repeated with the collected DNA in new tubes.

The collected DNA solution was cleaned by three extractions of 1x volume isopropanol (water/NaCl saturated) and desalted by dialysis against 2 litres 1 x TE buffer overnight (with one change of buffer during the dialysis).

2.10. Generation of transgenic mice.

2.10.1. Purification of DNA for pronuclear microinjection.

Procedures were based upon experimental protocols by Hogan et al., (1994). Although two types of driver construct were made, only the tTA containing construct was used. The tTA construct was excised from the cloning vector using a Not I
digestion (50 μg DNA) in a final reaction volume of 150 μl. Linearised DNA was cleaned (2 x P:C:I and 1 x chloroform extraction) with the P:C:I layer being re-extracted with 1 x TE buffer to increase the recovery of DNA. The DNA was ethanol precipitated and resuspended in 50 μl 1 x TE buffer. The DNA was loaded on to a 0.6 % agarose GTG gel and run at a speed of 1.5 V/cm. After approximately 6 hours, the marker lanes were stained with ethidium bromide and the whole gel was visualised under UV light. Care was taken not to expose the DNA fragment to UV light so only the marker bands were exposed. Once the fragment of the appropriate size (10 kb in this case) was located, it was cut out and the fragment was electroeluted from the gel slice using the Biotrap elution apparatus (Schleicher & Schuell) for at least 6 hours at 150 V in 1 x TAE buffer. The DNA solution was ethanol precipitated and desalted using 70 % ethanol and resuspended in Elutip low-salt buffer (Schleicher & Schuell). The DNA was purified through an Elutip column according to the manufacturers instructions (Schleicher & Schuell). The DNA was again desalted by using 70 % ethanol and then resuspended in sterile filtered 1 x TE buffer.

The DNA was stored at a concentration of 100 ng/μl at –80 °C until needed for microinjection.

2.10.2. Generation of founder mice.

The pronuclear microinjection of the tTA driver construct was carried out by Dr. Vasso Episkopou at the Hammersmith Hospital, London.

Briefly, single-cell fertilised eggs were obtained from superovulated 3 week old female mice mated the previous night. The following day, animals were culled by cervical dislocation. Oviducts were removed and single cell eggs were squeezed out of the ampulla. Eggs were then cleaned in hydrolurnidase + M2 medium (Sigma) (300 μg/ml) solution to remove attached cumulus cells and eggs were then left in M16 medium (Sigma) microdrops in a humidified incubator (37 °C, 5% CO₂, 95 % air) prior to injection.
Once eggs were injected, they were implanted by oviduct transfer into pseudopregnant female mice (approx. 15-20 injected eggs each side), under anaesthetic. Pups were usually delivered after 19 days gestation (where the night of the plug was E0).

2.11. Genotyping founder mice.

2.11.1. Extraction of genomic tail DNA.

Tail biopsies (approx. 1 cm of tail) of mice were taken once they reached 3-4 weeks old. Genomic tail DNA was obtained using a modified proteinase K digestion/isopropanol precipitation protocol (Laird et al., 1991). Tail tissue was incubated overnight in 500 µl tail lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, 100 µg/ml Proteinase K- Boehringer Mannheim). Samples were rotated slowly at 55 °C. The lysed tissue was vortexed for 1 minute and spun at 15,800g for 10 minutes to remove hair and bones. The supernatant was decanted into a new tube and to this, 500 µl isopropanol was added. The solutions were inverted until a white DNA precipitate could be seen. The DNA was pelleted, washed twice in 70 % ethanol and allowed to air-dry for a few minutes before it was resuspended in 100 µl 1 x TE buffer.

2.11.2. Southern blotting and PCR analysis.

Tail DNA from mice were first analysed by Southern blot using a 750 bp EcoRI-Sac II fragment from the tTA gene as a probe (Figure 3.26). Tail DNA (3-5 µg) was digested overnight with EcoRV (80 U) in a 100 µl reaction volume. Reactions were desalted before gel loading. Gels were run overnight at a speed of 1 V/cm in a 0.6 % agarose GTG gel made in 1 x TBE buffer. Blotting was carried out overnight using the capillary method in 10x SSPE (Sambrook et al., 1989). Prehybridisation,
hybridisation and washing of hybridised blots was carried out as described previously. As an additional confirmation, DNA giving positive signals from the exposed X-ray films were screened using a PCR protocol developed by Mohammed Nassar, UCL. The PCR mix was composed of the following (total volume 25 μl):

1 μl 1:5 (~1 μg) dilution of genomic DNA in water
1 μl mNR1-Seq38-s primer (10 μM)
1 μl GluN1-Seq3-a primer (10 μM)
1 μl TA-Seq-2-a primer (10 μM)
1 μl 5 mM nucleotide mix
2.5 μl 10 x Taq polymerase buffer (Promega)
17.3 μl Milli-Q water
0.2 μl Taq polymerase, 5U/μl (Promega)

PCR conditions were as follows; Initial denaturation 94 °C, 5 minutes; Denaturation 94 °C, 30 seconds; Annealing 60 °C, 30 seconds; Extension 72 °C, 45 seconds; 30 cycles. The following primers were used:

ACCAGTGCACAGTCCAGGCAGCT-mNR1-Seq38-s (5'-3')

GGCGTTGAGCTGTATCTTCC- GluN1-Seq3-a (5'-3')

CTAGCTTTCTGGCGAGTTACGGGT- TA-Seq2-a (5'-3')

The three primer PCR, generated two fragments from DNA from mice containing the tTA transgene. A 400 bp wildtype fragment was generated from mNR1-Seq38-s and GluN1-Seq3-a while a 300 bp fragment from the junction of the mNR1 promoter and the tTA gene was generated from the mNR1-Seq38-s and TA-Seq2-a combination. PCR samples were analysed in a 2 % agarose LE gel in 1 x TAE buffer.
3. Results.

3.1. Overview.

*Experiments to identify amino acid residues controlling glutamate potency on recombinant NMDA receptors*- Point mutations were inserted into NR2A expression vectors at sites within the S1 and S2 regions using PCR based strategies (Figures 3.3-3.5). Mutations were confirmed by restriction enzyme digestion and DNA sequencing.

Wildtype NR1 and mutant NR2A cRNA transcripts were co-expressed in *Xenopus* oocytes. Glutamate-evoked inward currents (in the presence of 30 µM glycine) were recorded in two-electrode voltage clamp configuration (Figure 3.6).

Glutamate and glycine dose-response curves were measured for each NR1/mutant NR2A combination and the $EC_{50}$s were compared to wildtype values (Figures 3.7-3.8 and Table 4).

To confirm that any reduction in glutamate potency was due to a disruption in glutamate binding, the actions of two competitive antagonists on the NR2A(T671A) mutation were studied. The dissociation equilibrium binding constants, $K_B$, for the glutamate site antagonists, APV and R-CPP were measured using Schild analysis (Figures 3.9-3.12).

To investigate the effect of two of these mutations on recombinant NR1/NR2D receptors, two mutations were made in the NR2D subunit (T692A and K486E) which are homologous to positions T671 and K465 in the NR2A subunit (Figure 3.13). Mutations were again generated by PCR and confirmed by DNA sequencing. Glutamate and glycine dose-response curves were measured for wildtype NR1/NR2D receptors and both NR2D mutants (Figures 3.14-3.15 and Table 5).

*Experiments to identify the NR2A copy number in recombinant NR1/NR2A NMDA receptors*- Using a similar strategy described by Laube et al., (1998), an attempt was made to count the number of NR2 subunits in recombinant NR1/NR2B receptors (see 1.2.4). Wildtype and mutant NR2A cRNAs were coexpressed to exploit the differences in glutamate potency seen between receptors containing either NR2A
subunit. The NR2A and NR2A(T671A) cRNAs were coexpressed in different ratios to identify the number of intermediate channel species formed. This was done by identifying the number of different components (wildtype, intermediates or mutant) that could be fitted to the glutamate dose-response curve (Figures 3.16-3.21 and Table 6). The cRNAs were mixed in 2:1 and 1:2 ratios and co-injected, with NR1 cRNA.
Generation of a transgenic mouse containing elements to drive the tetracycline-controlled transactivator (tTA) in the brain. Restriction enzyme digestion and Southern analysis revealed approximately 8 kb of 5'UT region in a λ-phage clone containing the mouse NMDA NR1 gene (Figure 3.22). Further mapping identified suitable restriction enzyme sites which could be used for subcloning the 8 kb fragment (Figure 3.23). This 5'UT region was placed 5' upstream of the tTA gene to generate a 10 kb 'driver' construct. Further subcloning steps inserted two transcriptional stop sites flanking the transgene to prevent position dependent effects which may occur once the transgene has integrated into the genome (Figures 3.24-3.26).

The transgene was excised from the plasmid vector using two flanking Not I sites and purified to remove contaminant proteins and particulate matter. The purified transgene was injected into single cell mouse eggs to generate transgenic founder mice. These eggs were embryo transferred into pseudopregnant foster mice. Pups were born after three weeks gestation and were genotyped for the injected transgene after another three weeks (after weaning).

Initial screening for transgenic founders was done by Southern analysis using a tTA radiolabelled probe (Figure 3.27). Positive founders were additionally confirmed by PCR.

Figure 3.1. Diagram showing the components assembled for the driver construct. Boxes representing components are not to scale. Arrow indicates transcriptional start site.
3.1.1. Generation of NR2A point mutations.

Alignment of NR2 sequences with bacterial amino acid binding proteins showed limited sequence identity in the S1 and S2 segments (Figure 3.2). The ten mutations were chosen either because they were known to control glycine potency on the NR1 subunit or that they control amino acid binding in bacterial binding proteins. Generally uncharged amino acids were mutated to an alanine, charged residues were changed to those of opposite charge and aromatic residues were replaced by residues with similar side chains. All mutants were generated by site-directed mutagenesis using PCR based strategies as described in the Methods. Plasmid maps in Figures 3.3-3.5. show the construction of the 10 mutants.
Figure 3.2. Amino acid sequence alignment of rat NR1 and NR2 subunits with the bacterial periplasmic binding proteins LAOBP and HISJ. Residues that reduced glycine potency when mutated in NR1 are marked by an arrow above the sequence alignment. NR2A residues that were mutated in this study are marked below the sequence alignments either as an arrow if the mutation reduced potency by more than a factor of 2, otherwise by a -. Numbers indicate the first residue of the alignment. From Anson et al., (1998).
Figure 3.3. Plasmid maps showing the construction of the NR2A(N463A), NR2A(K465E), NR2A(H466A) and NR2A(H466F) mutations. Cloning enzymes are in bold, and diagnostic enzymes introduced with the PCR fragment are underlined. The introduced PCR fragment is represented by stripes. The small arrows show the general location of the PCR primers (not to scale). The dashed arrow indicates the general location of the point mutation. The thick circle within the plasmid represents the wildtype NR2A subunit cDNA coding region.
Figure 3.4. Plasmid maps showing the construction of the NR2A(G669A), NR2A(S670A), NR2A(T671A) and NR2A(V666A) mutations. Cloning enzymes are in bold, and diagnostic enzymes introduced with the PCR fragment are underlined. The introduced PCR fragment is represented by stripes. The small arrows show the general location of the PCR primers (not to scale). The dashed arrow indicates the general location of the point mutation. The thick circle within the plasmid represents the wildtype NR2A subunit cDNA coding region.
Figure 3.5. Plasmid maps showing the construction of the NR2A(G664A) and NR2A(T665A) mutations. Cloning enzymes are in bold, and diagnostic enzymes introduced with the PCR fragment are underlined. The introduced PCR fragment is represented by stripes. The small arrows show the general location of the PCR primers (not to scale). The dashed arrow indicates the general location of the point mutation. The thick circle within the plasmid represents the wildtype NR2A subunit cDNA coding region.
3.1.2. Recombinant coexpression of NR1/NR2 (mutant) receptors.

All ten NR2A mutants, when coexpressed with NR1 cRNA, in oocytes produced robust inward currents upon application of glutamate in the presence of glycine. Injection of similar amounts of RNA generally produced maximal inward currents not lower than wildtype (Figure 3.6)(Table 3).

<table>
<thead>
<tr>
<th>NR1/NR2A combination</th>
<th>Mean glutamate evoked maximum inward current, $I_{\text{max}} \pm SDM$ (nA)</th>
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<tbody>
<tr>
<td>Wildtype</td>
<td>406 ± 92</td>
</tr>
<tr>
<td>N463A</td>
<td>1307 ± 370</td>
</tr>
<tr>
<td>K465E</td>
<td>1599 ± 433</td>
</tr>
<tr>
<td>H466A</td>
<td>1283 ± 231</td>
</tr>
<tr>
<td>H466F</td>
<td>1246 ± 479</td>
</tr>
<tr>
<td>G664A</td>
<td>761 ± 393</td>
</tr>
<tr>
<td>T665A</td>
<td>795 ± 199</td>
</tr>
<tr>
<td>V666A</td>
<td>1656 ± 872</td>
</tr>
<tr>
<td>G669A</td>
<td>3747 ± 383</td>
</tr>
<tr>
<td>S670A</td>
<td>382 ± 70</td>
</tr>
<tr>
<td>T671A</td>
<td>2035 ± 469</td>
</tr>
</tbody>
</table>

Table 3. Mean glutamate evoked maximum inward currents from oocytes injected with the corresponding NR1/NR2A combination. Values were taken from the fitted glutamate dose response curves in Figure 3.7.
Figure 3.6. Glutamate activated inward currents recorded in the two-electrode voltage clamp configuration from oocytes expressing NR1/NR2A wild-type or NR1/NR2A(T671A) receptors. Recordings made in the presence of 30 μM glycine by Dr. L. C. Anson. From Anson et al., (1998).
3.2. Agonist dose-response curves.

Measurement of glutamate and glycine dose-response curves for the wildtype and mutant NMDA receptors were done in collaboration with Dr. Lesley Anson who shares the credit for making these measurements (The glutamate dose-response curves for NR2A(H466A), NR2A(G669A), NR2A(T671A) and NR2A(G664A) and the glycine dose-response curves for NR2A(V666A), NR2A(T665A) and NR2A(G664A)).

3.2.1. Glutamate dose-response curves.

Wildtype NR1/NR2A receptors produced fitted dose-response curves which gave a glutamate EC$_{50}$ similar to the values obtained by others (~3.0 μM-see Table 2). Fitted dose response curves showed that all the mutants (except NR2A(G664A)) produced rightward shifts in the glutamate dose-response curves compared those for wildtype NR1/NR2A (Figure 3.7. and Table 4.). Of these, only NR2A(S670A) did not produce a decrease in glutamate potency of more than five fold compared to wildtype. However, three mutants reduced glutamate potency by more than 100-fold compared to wildtype. Two of these mutants, NR2A(H466A), and NR2A(G669A) produced shifts in glutamate EC$_{50}$ of more than 100 fold (216- and 321-fold respectively). However, change of the histidine residue at position 466 to an aromatic phenylalanine produced only a 10-fold change in glutamate potency compared to wildtype, suggesting that bulky side chain groups (imidazole or aromatic) may be important for controlling glutamate potency at this position. The NR2A(T671A) mutation produced a reduction in glutamate potency of more than a 1000-fold (1027-fold). The change in charge at position 465 from positive (lysine) to negative (glutamic acid) produced only a 10-fold change in glutamate potency compared to wildtype. There was little change in Hill coefficient calculated from the fitted dose-response curves among most of the mutants compared to wildtype NR1/NR2A dose-response curves (especially from the three which gave the biggest shifts in glutamate EC$_{50}$).
However a few mutants produced dose response curves with Hill coefficients that may be higher (K465E) or lower (V666A) than NR1/NR2A wildtype values (Table 4).

3.2.2. Glycine dose-response curves.

The wildtype NR1/NR2A glycine EC$_{50}$ was also similar to values obtained previously by others (Table 2). The saturating glutamate concentrations used for each NR2A mutant were as follows: NR2A(G664A) and NR2A(S670A), 30 µM; NR2A(N463A), 50 µM; NR2A(K465E), NR2A(H466F), NR2A(T665A) and NR2A(V666A), 300 µM; NR2A(H466A), 3000 µM; NR2A(G669A) and NR2A(T671A), 10000 µM. In contrast to the glutamate dose-response curves, there was little difference in glycine potency between the glycine dose response curves of the ten mutants and that of wildtype NR1/NR2A receptors (Figure 3.8). The glycine EC$_{50}$ values of the ten mutants lay within a factor of 1.7 of each other with little change in Hill coefficient between them (Table 4).
Figure 3.7. Mean normalised glutamate dose response curves for wild-type (filled circles), N463A (open squares), K465E (open diamonds), H466A (inverted filled triangles) H466F (filled triangles), G664A (open triangles), T665A (filled diamonds), V666A (crosses), G669A (open circles), S670A (inverted open triangles) and T671A (filled squares) receptors fitted with the Hill equation. From Anson et al., (1998).
Figure 3.8. Mean normalised glycine dose response curves for wild-type (filled circles), N463A (open squares), K465E (open diamonds), H466A (inverted filled triangles), H466F (filled triangles), G664A (open triangles), T665A (filled diamonds), V666A (crosses), G669A (open circles), S670A (inverted open triangles) and T671A (filled squares) receptors fitted with the Hill equation. From Anson et al., (1998).
Glutamate and glycine EC$_{50}$ and Hill values for the wildtype and the ten mutant combinations.

<table>
<thead>
<tr>
<th></th>
<th>Glutamate</th>
<th>Glycine</th>
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<tbody>
<tr>
<td></td>
<td>EC$_{50}$ (µM)</td>
<td>nH</td>
</tr>
<tr>
<td>NR1/NR2A</td>
<td>2.89 ± 0.12</td>
<td>1.49 ± 0.09</td>
</tr>
<tr>
<td>NR1/NR2A(N463A)</td>
<td>17.9 ± 1.4</td>
<td>1.70 ± 0.18</td>
</tr>
<tr>
<td>NR1/NR2A(K465E)</td>
<td>29.3 ± 1.4</td>
<td>1.91 ± 0.18</td>
</tr>
<tr>
<td>NR1/NR2A(H466A)</td>
<td>624 ± 32</td>
<td>1.55 ± 0.09</td>
</tr>
<tr>
<td>NR1/NR2A(H466F)</td>
<td>38.4 ± 4.6</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>NR1/NR2A(G664A)</td>
<td>2.08 ± 0.25</td>
<td>1.46 ± 0.21</td>
</tr>
<tr>
<td>NR1/NR2A(T665A)</td>
<td>19.0 ± 2.0</td>
<td>1.18 ± 0.11</td>
</tr>
<tr>
<td>NR1/NR2A(V666A)</td>
<td>33.4 ± 1.6</td>
<td>1.12 ± 0.05</td>
</tr>
<tr>
<td>NR1/NR2A(G669A)</td>
<td>927 ± 46</td>
<td>1.38 ± 0.07</td>
</tr>
<tr>
<td>NR1/NR2A(S670A)</td>
<td>4.97 ± 0.30</td>
<td>1.35 ± 0.09</td>
</tr>
<tr>
<td>NR1/NR2A(T671A)</td>
<td>2967 ± 279</td>
<td>1.30 ± 0.09</td>
</tr>
</tbody>
</table>

Table 4. Glutamate and glycine EC$_{50}$ and Hill coefficients calculated from the pooled dose-response curves (Figures 3.7 and 3.8). Values are given as means ± S.D.M.
3.3. Measurement of APV and R-CPP affinity on wildtype and mutant receptors.

3.3.1. Schild analysis of the NR1/NR2A wildtype and NR1/NR2A(T671A) mutant receptors.

Partial, low concentration glutamate dose response curves in the absence and presence of the competitive glutamate site antagonist, APV are shown in Figure 3.9. These were measured from wildtype NR1/NR2A recombinant receptors and NR1/NR2A(T671A) mutant receptors. The T671A mutation was chosen to see if the large reduction in glutamate potency observed with this mutation would cause any change in the affinity of a competitive antagonist at the glutamate binding site. The dissociation equilibrium binding constant for the antagonist was measured as \( K_b \) using the Schild method. The slope of the Schild plot from five separate oocytes was 1.06 ± 0.04 for wildtype NR1/NR2A receptors and 0.94 ± 0.16 for NR1/NR2A(T671A) receptors (Figure 3.10). These values are very close to one, suggesting that APV is acting as a competitive antagonist not only on wildtype receptors but also on NR1/NR2A(T671A) mutant receptors. The Schild plot was then refitted with a slope of unity (as defined by the Schild equation) and the \( K_b \) was calculated from the intercept of the plot in the x-axis. For the wildtype NR1/NR2A receptors the \( K_b \) was 1.26 ± 0.07 \( \mu \)M which is similar to the values found by (Verdoorn et al., 1989) from oocytes injected with rat brain mRNA. However for the NR1/NR2A(T671A) mutant the \( K_b \) was 321 ± 30 \( \mu \)M. Thus the mutant had a 255-fold reduction in affinity for APV compared to wildtype.
Figure 3.9. Partial dose-response curves for glutamate in the absence and presence of APV. A, Partial, low concentration, glutamate dose-response curves for one oocyte expressing wild-type receptors in the absence (open squares) and presence of 3 mM (filled diamonds), 10 mM (open pentagons), 30 mM (filled hexagons) and 100 mM (open circles) APV. Dashed lines represent free fits of the power function to the data, and solid lines show fits of the same function but with slopes constrained to be the same for all curves. Lines have been extrapolated to show the similarities between the fitted dashed and solid lines. B, Partial, low concentration, glutamate dose-response curves for one oocyte expressing T671A mutant receptors in the absence (filled pentagons) and presence of 300 µM (open diamonds) and 1 mM (filled triangles) APV. Data fitted as in A. From Anson et al., (1998).
Figure 3.10. Schild plot for competitive antagonism of wild-type (filled circles) and T671A (filled squares) receptors by APV. Data obtained from dose-ratios estimated from results such as those in Figure 3.9. The points represent the means of 5 dose ratios. The dashed lines are free fits with slopes that are not exactly 1; the solid lines are fits of the Schild equation (both have slope = 1). Fitted lines have been extrapolated for display purposes. From Anson et al., (1998).
3.3.2. Schild analysis for the antagonism of NR1/NR2A and NR1/NR2A(T671A) receptors by R-CPP.

Partial glutamate dose-response curves show that R-CPP shifts the dose-response curve to the left in a parallel fashion in both NR1/NR2A and NR1/NR2A(T671A) receptors (Figure 3.11). The dose-ratios generated in the presence of increasing concentrations of CPP, were plotted as log[dose-ratio-1] vs log[CPP]. The Schild plot could be fitted with a slope of unity for both receptors suggesting that the data follows the Schild equation (Figure 3.12) and that CPP acts competitively on NR1/NR2A receptors (Slope = 0.94 ± 0.06) and NR1/NR2A(T671A) receptors (Slope = 1.03 ± 0.12). After fitting the data with a slope of unity, the dissociation equilibrium binding constant $K_B$, for R-CPP, was found to be 0.31 ± 0.01 μM on recombinant NR1/NR2A receptors. However, there was an 82-fold reduction in R-CPP affinity in NR1/NR2A(T671A) receptors ($K_B = 24.6 ± 2$ μM) compared to wild-type NR1/NR2A receptors. This change is 3-fold smaller than the reduction in APV affinity found on NR1/NR2A(T671A) receptors (section 3.3.1.).
Figure 3.11. Partial dose-response curves for glutamate in the absence and presence of CPP. A, Partial, low concentration, glutamate dose-response curves for one oocyte expressing wild-type receptors in the absence (filled squares) and presence of 1 µM (open diamonds), 3 µM (filled pentagons), 10 µM (open hexagons) and 30 µM (filled circles) CPP. Solid lines represent fits of the power function to the data with slopes constrained to be the same for all curves. B, Partial, low concentration, glutamate dose-response curves for one oocyte expressing T671A mutant receptors in the absence (filled squares) and presence of 30 µM (open diamonds) and 100 µM (filled pentagons) CPP. Data fitted as in A. C and D are fits of the same data where the spacing of the fits has been defined by the Schild equation. This would correspond to a Schild plot with a slope = 1 and allows $K_B$ to be estimated without the need to do a Schild plot. For C, the calculated $K_B = 0.31 \pm 0.03$ µM and for D, $K_B = 24.3 \pm 2.6$ µM. Both values are close to those calculated from the Schild plot in Figure 3.12.
Figure 3.12. Schild plot for competitive antagonism of wild-type (filled squares) and T671A (filled circles) receptors by R-CPP. Data obtained from dose-ratios estimated from results such as those in Figure 3.11. The points represent the means of 4 dose ratios. The dashed lines are free fits with slopes that are not exactly 1; the solid lines are fits of the Schild equation (both have slope = 1). Fitted lines have been extrapolated for display purposes.
3.4. Expression of NR1/NR2D mutant receptors.

Plasmid maps of the two NR2D mutants are shown in figure 3.13. These are at analogous positions to the NR2A(T671) and NR2A(K465) residues in the NR2A subunit. Both NR1/NR2D mutants expressed robust inward currents in the presence of glutamate (and 30 μM glycine). The mean maximal currents for NR1/NR2D(T692A) and NR1/NR2D(K486E) receptors were 226 ± 73 nA and 477 ± 51 nA respectively. The NR1/NR2D wild-type combination expressed with similar efficiency (mean maximal current = 147 ± 35 nA).

3.4.1. Glutamate dose-response curves of NR1/NR2D, NR1/NR2D(T692A) and NR1/NR2D(K486E) receptors.

Glutamate dose-response curves were generated for both NR2D mutant receptors (Figure 3.14)(Table 5). For the NR1/NR2D(T692A) combination, the glutamate potency was reduced by about 1800-fold (glutamate $EC_{50} = 804 ± 68 \mu M$) compared to wild-type NR1/NR2D receptors (glutamate $EC_{50} = 0.45 ± 0.04 \mu M$). There was little difference between the Hill slopes of either receptor combination (NR1/NR2D receptors, $n_H = 1.31 ± 0.15$, NR1/NR2D(T692A) receptors, $n_H = 1.41 ± 0.15$). This is similar to what was observed for NR1/NR2A(T671A) receptors. However, the glutamate potency of NR1/NR2D(K486E) receptors was only reduced by 34-fold compared to wild-type (glutamate $EC_{50} = 15.5 ± 1.3 \mu M$, $n_H = 1.65 ± 0.20$). This is slightly larger than the shift found for NR1/NR2A(K465E) receptors (only 10-fold reduction).
3.4.2. Glycine dose-response curves of NR1/NR2D, NR1/NR2D(T692A) and NR1/NR2D(K486E) receptors.

NR1/NR2D(T692A) and NR1/NR2D(K486E) glycine dose-response curves were generated in the presence of saturating concentrations of glutamate (10 mM and 100 μM respectively). Both NR2D mutations had little effect on glycine potency (Figure 3.15)(Table 5). The NR1/NR2D(T692A) glycine $EC_{50}$ differed by less than two-fold compared to wildtype and the NR1/NR2D(K465E) glycine $EC_{50}$ differed by less than four-fold. Both results are very different from the effects on glutamate potency. Thus, the results are similar to those found for analogous mutations in the NR2A subunit (see section 3.2.).

<table>
<thead>
<tr>
<th>NR1/NR2D combination</th>
<th>Glutamate $EC_{50}$ (μM)</th>
<th>Hill slope, $n_H$</th>
<th>n</th>
<th>Glycine $EC_{50}$ (μM)</th>
<th>Hill slope, $n_H$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1/ NR2D WT</td>
<td>0.45 ± 0.04</td>
<td>1.31 ± 0.15</td>
<td>5</td>
<td>0.11 ± 0.01</td>
<td>1.51 ± 0.19</td>
<td>4</td>
</tr>
<tr>
<td>NR1/ NR2D(T692A)</td>
<td>804 ± 68</td>
<td>1.41 ± 0.15</td>
<td>5</td>
<td>0.08 ± 0.01</td>
<td>1.19 ± 0.13</td>
<td>4</td>
</tr>
<tr>
<td>NR1/ NR2D(K486E)</td>
<td>15.5 ± 1.3</td>
<td>1.65 ± 0.2</td>
<td>4</td>
<td>0.28 ± 0.03</td>
<td>1.22 ± 0.15</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5. Glutamate and glycine $EC_{50}$s and Hill coefficients from pooled dose-response curves of NR1/NR2D, NR1/NR2D(T692A) and NR1/NR2D(K486E) receptors. Values are means ± SDM.
Figure 3.13. Plasmid maps showing the construction of the NR2D(T692A) and NR2A(K486E) mutations. Cloning enzymes are in bold, and diagnostic enzymes introduced with the PCR fragment are underlined. The introduced PCR fragment is represented by stripes. The small arrows show the general location of the PCR primers (not to scale). The dashed arrow indicates the general location of the point mutation. The thick circle within the plasmid represents the wildtype NR2D subunit cDNA coding region. For the K486E mutation, the primers are numbered to show which PCR product they correspond to. The 'outside' primers are marked with an asterisk—see (2.4.6).
Figure 3.14. Mean normalised glutamate dose response curves for NR1/NR2D wild-type (filled squares), NR1/NR2D(K486E) (open diamonds) and NR1/NR2D(T692A) (filled pentagons) receptors fitted with the Hill equation.
Figure 3.15. Mean normalised glycine dose response curves for NR1/NR2D wild-type (filled squares), NR1/NR2D(K486E) (open diamonds), NR1/NR2D(T692A) (filled pentagons) receptors fitted with the Hill equation.
3.5. Coexpression of NR1/NR2A/NR2A(T671A) cRNA mixtures.

To identify the number of intermediate components (and thus receptor species) formed between mixtures of wildtype and mutant NR2A subunits, glutamate dose-response curves were measured from oocytes injected with NR2A and NR2A(T671A) cRNAs mixed in different ratios. Glutamate dose-response curves for mixtures of NR1/NR2A/NR2A(T671A) cRNAs in a ratio of 1:2:1 could be fitted with a single-component Hill equation (Figure 3.16). However the glutamate $EC_{50}$ differed from either NR1/NR2A receptors or NR1/NR2A(T671A) receptors ($glutamate EC_{50} = 50 \pm 4 \mu M, n_H = 1.54 \pm 0.15, n = 6$). The data could also be fitted with a biphasic dose-response curve, (Figure 3.17) if the Hill coefficient was fixed at an extremely high value (6 in this case).

For the NR1/NR2A/NR2A(T671A) 1:1:2 mix, a triphasic glutamate dose-response curve could be fitted. One intermediate dose-response curve could be seen (Figure 3.18). The glutamate $EC_{50}$ for this intermediate component was $72 \pm 7 \mu M$. The other two components of the dose-response curve had $EC_{50}$s similar to all wildtype and all mutant receptors (Figure 3.18 and Table 7). However, the Hill slopes for the components were slightly greater than the all wild-type and all mutant dose response curves (see Table 7). This was particularly clear for the first component with a glutamate $EC_{50}$ of $4.4 \mu M$. If the 1st and 3rd components were fixed to glutamate $EC_{50}$s and Hill slopes for the all wildtype and all mutant receptors (Table 7), the 2nd component could be fitted with a glutamate $EC_{50} = 61 \pm 11 \mu M$ and a Hill slope of $1.25 \pm 0.17$, which was closer to the values obtained from the predominant component obtained from the 1:2:1 mixture (Figure 3.19). Therefore, there is at least one intermediate component present and this is observed in both the 2:1 and 1:2 NR2A /NR2A(T671A) mixtures. It is assumed this component is composed of receptors containing at least one wildtype and one mutant NR2A(T671A) subunit. Figure 3.20 shows the data fitted with a 3-component curve where the $EC_{50}$s and the Hill slopes
were fixed to those from all wildtype receptors, all mutant receptors and the values obtained from oocytes injected with NR2A:NR2A(T671A) cRNA in a 2:1 ratio (as the intermediate). It is unclear why the 1:2:1 combination produced a predominantly uni-component dose-response curve whereas the 1:1:2 combination was clearly triphasic. Furthermore an attempt was made to fit a 4-component dose-response to the 1:2 NR2A/NR2A(T671A) data, by fixing the 1st and 4th components to all wildtype and all mutant values (Figure 3.21). However, again some Hill coefficients were extremely high (e.g. 4.43 for the 3rd component-see Table 7). Nevertheless, it was impossible to determine whether the data were better fit with a 3- or 4- component curve (Figure 3.21).

<table>
<thead>
<tr>
<th>Mixture type</th>
<th>Mean Glutamate ( EC_{50} ) (( \mu \text{M} ))</th>
<th>Hill slope, ( n_H )</th>
<th>Fraction of normalised response</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>All NR1/NR2A Wild-type*</td>
<td>2.89 ± 0.12</td>
<td>1.49 ± 0.09</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>NR1/NR2A/NR2A(T671A) 1:2:1 1-component fit (Figure 3.16)</td>
<td>50 ± 4</td>
<td>1.54 ± 0.15</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>2-component fit (Figure 3.17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st component</td>
<td>50</td>
<td>1.67</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>2nd component</td>
<td>1845</td>
<td>6.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>All NR1/NR2A (T671A) Mutant*</td>
<td>2967 ± 279</td>
<td>1.3 ± 0.09</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 6. Glutamate \( EC_{50} \)s and Hill coefficients from oocytes injected with NR2A and NR2A(T671A) cRNAs in a 2:1 ratio. Values are means ± SDM. *The all wild-type NR1/NR2A and all mutant NR1/NR2A(T671A) glutamate \( EC_{50} \)s were taken from Table 4.
<table>
<thead>
<tr>
<th>Mixture type</th>
<th>Mean Glutamate $EC_{50}$ (µM)</th>
<th>Hill slope, $n_H$</th>
<th>Fraction of normalised response</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>All NR1/NR2A Wild-type*</td>
<td>2.89 ± 0.12</td>
<td>1.49 ± 0.09</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>NR1/NR2A/NR2A(T671A) 1:1:2 (Figure 3.18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-component free fit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st component</td>
<td>4.4 ± 1.2</td>
<td>2.0 ± 0.27</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>2nd component</td>
<td>72 ± 7</td>
<td>2.0 ± 0.47</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>3rd component</td>
<td>2071 ± 206</td>
<td>1.7 ± 0.29</td>
<td>47</td>
<td>4</td>
</tr>
<tr>
<td>Constrained fit of 1st and 3rd components, free fit of 2nd component (Figure 3.19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st component</td>
<td>2.89</td>
<td>1.49</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>2nd component</td>
<td>61 ± 11</td>
<td>1.25 ± 0.17</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3rd component</td>
<td>2967</td>
<td>1.30</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Constrained fit of 1st and 4th components, free fit of 2nd and 3rd components (Figure 3.21)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st component</td>
<td>2.89</td>
<td>1.49</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2nd component</td>
<td>62 ± 25</td>
<td>1.25 ± 0.3</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3rd component</td>
<td>1666 ± 2270</td>
<td>4.43 ± 7</td>
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</tr>
<tr>
<td>4th component</td>
<td>2967</td>
<td>1.30</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Constrained fit of all 3 components (Figure 3.20)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1st component</td>
<td>2.89</td>
<td>1.49</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>2nd component</td>
<td>50</td>
<td>1.54</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>3rd component</td>
<td>2967</td>
<td>1.30</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>All NR1/NR2A (T671A) Mutant*</td>
<td>2967 ± 279</td>
<td>1.3 ± 0.09</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 7. Glutamate $EC_{50}s$ and Hill coefficients from oocytes injected with NR2A and NR2A(T671A) cRNAs in a 1:2 ratio. Values are means ± SDM, *The all wild-type NR1/NR2A and all mutant NR1/NR2A(T671A) glutamate $EC_{50}s$ were taken from Table 4.
Figure 3.16. Mean normalised glutamate dose response curves from oocytes injected with NR1/NR2A/NR2A(T671A) cRNAs in a 1:2:1 ratio. The all NR1/NR2A wild-type (filled pentagons), NR1/NR2A/NR2A(T671A) 1:2:1 ratio (open diamonds) and all NR1/NR2A(T671A) receptors (open hexagons) were fitted with the Hill equation. All NR1/NR2A wild-type and all NR1/NR2A(T671A) data were taken from Anson et al., (1998) and are shown for comparison.
Figure 3.17. A 2-component fit of the NR1/NR2A/NR2A(T671A) 1:2:1 data from Figure 3.16. The curve was forced to give a 2-component fit with the 1st and 2nd components described by glutamate $EC_{50}$s of 50 and 1845 $\mu$M respectively.
Figure 3.18. Mean normalised glutamate dose response curve from oocytes injected with NR1/NR2A/NR2A(T671A) cRNAs in a 1:1:2 ratio. The all NR1/NR2A wild-type (filled pentagons), NR1/NR2A/NR2A(T671A) 1:1:2 ratio (filled squares), all NR1/NR2A(T671A) receptors (open hexagons) fitted with either a single component or three-component form of the Hill equation. All NR1/NR2A wild-type and all NR1/NR2A(T671A) data were taken from Anson et al., (1998) and are shown for comparison.
Figure 3.19. Free-fit of the 2nd component of the NR1/NR2A/NR2A(T671A) 1:1:2 data. The data was fitted with a curve (blue line) where the \( EC_{50} \) and Hill slopes of the 1st and 3rd components are fixed to either the wildtype NR1/NR2A or NR1/NR2A(T671A) values. The 2nd component was left as a free fit, giving a glutamate \( EC_{50} = 61 \mu M \) and a Hill slope of 1.25. The red dashed line is the free fit described in Figure 3.18.
Figure 3.20. Three-component constrained fit of the NR1/NR2A/NR2A(T671A) 1:1:2 data. The data was fitted with a curve (blue line) where the $EC_{50}$s and Hill slopes of the 1st, 2nd and 3rd components are fixed to either the wildtype NR1/NR2A, NR1/NR2A/NR2A(T671A) 1:2:1 mix and NR1/NR2A(T671A) values (see Table 6). The red dashed line is the free fit described in Figure 3.18.
Figure 3.21. Three and four-component fits of the NR1/NR2A/NR2A(T671A) 1:1:2 data. The 1st and 3rd components for the 3-component fit and the 1st and 4th components for the 4-component fit are constrained to either all wild-type or all mutant values, leaving the intermediate components as free fits (as in Figure 3.19). The 3-component fit is shown in blue and the 4-component fit is represented by a green dashed line.
3.6. Construction of the NR1-tTA 'driver' construct.

3.6.1. Mapping and subcloning the 5' UT region into bluescript.

Initial digestion of the 36.1. λ-phage clone known to contain the 5'UT region and exon 1 of the mouse NMDA NR1 gene and subsequent Southern blot hybridisation (using an EcoR I-Nde I probe from a previously mapped section of the 5'UT region from Mohammed Nassar-UCL) (see Figure 3.22) revealed that approximately 8 kb of 5'UT region was present within the clone as a Not I-Sac II fragment (Figure 3.22). This was excised and inserted into an SKII- Bluescript vector for further mapping, to identify other restriction enzyme sites in this fragment (Figure 3.23). The Sac II site was just 5' of the ATG in exon 1 and the Not I site was from the junction of the inserted genomic fragment and the left arm of the λ-phage vector.

However, this fragment still contained undesirable DNA elements (from Not I-Sal I) at the furthermost 5' end of the region (a flanking T3 promoter for sequencing genomic DNA inserts). Thus, a Sal I digestion would be needed to maximise the length of 5'UT used in the driver construct and to remove the T3 region from the fragment. More detailed mapping revealed that another Sal I site was 600 bp downstream from the 5' most end of the fragment (Not I site). A Sal I digestion would shorten the 5'UT region used for the construction of the driver. For ease of cloning, the 5'UT region used in the driver covered a region from Sal I to Sac II with the 5'most 600 bp region absent (Sal I-Sal I) (Figure 3.23).

3.6.2. Subcloning- Driver construction.

The driver constructs were constructed using both tTA and rtTA genes, although only the driver containing the tTA gene was used for pronuclear microinjection. Each driver contained a 8 kb 5'UT region from the mouse NR1 gene placed upstream of either transactivator gene (tTA/rtTA). Cloning was carried out in a
pRSSP (Dr. Ralf Schoepfer, UCL) cloning vector using the methods described previously. The next steps describe the construction process (Figures 3.24-3.25).

Step 1. A polylinker containing the relevant cloning sites was designed and cloned into a pRSSP vector using Sac II-Mlu I digestion sites. Multiple cloning sites were present in the following order: Sac II-Pst I-Kpn I-Xho I-EcoR I-Bam HI-Hind III-Mlu I. A Not I site was downstream from the Mlu I site in the pRSSP vector and was required for excision of the driver construct from the vector backbone for pronuclear microinjection.

Step 2. The tTA fragment and the attached transcriptional stop signal (SV40 late poly(A) signal) was taken from the driver vector pUHD15-1 (Gossen and Bujard, 1992) as a EcoR I-Hind III fragment and cloned into the available sites in the polylinker.

Step 3. An EcoR I-Bam HI fragment from the vector pUHG17-l (Gossen et al., 1995) containing the rtTA gene was removed and cloned into the polylinker. Replacing the tTA cloned earlier. From this point identical cloning was done in either of the tTA/rtTA drivers.

Step 4. A synthetic upstream transcriptional stop signal was generated by PCR from the pGL3 (Promega) luciferase expression vector. The primers polyA-PCR3-s (sense) and polyA-PCR4-a (antisense) were used. The PCR conditions were; Initial denaturation 94 °C, 2 minutes; Denaturation 94 °C, 30 seconds; Annealling 55 °C, 15 seconds; Extension 72 °C, 30 seconds; 20 cycles.

gcgetgcAGCGGCCGCAATAAAAAATATCTTTA- polyA-PCR-3s (5'-3')

\textit{PstI} \hspace{4cm} \textit{NolI}

gcgetcgagTATCGATAGAAATGTCTGG- polyA-PCR-4a (5'-3')

\textit{XhoI}
Figure 3.22. Southern blot of digested lambda-phage DNA (36.1). The clone contained NR1 exon 1 and a region of unknown length 5' upstream from the ATG. An EcoR I-Nde I radiolabelled probe was used from the previously mapped mouse NR1 5' upstream region. A single Sac II site was present in the 5' untranslated region of exon 1 and the 5' arm of the lambda phage clone began with a Not I site. A Not I-Sac II double digest revealed that a single band was present about 7-8 kb upstream of the Sac II site in exon 1. The left lane contained 90% of the digestion and the right lane contained 10%. The blot was exposed for 10 minutes.
Figure 3.23. Restriction enzyme map of the 5' UT region from the mouse NR1 gene. The map shows the 8 kb 5'UT fragment isolated from a mouse genomic DNA library. Filled boxes represent exons. The filled arrow shows the transcriptional start site. Apa I= A, Bgl II= B, Bsu36 I= Bs, Cla I= C, EcoR I= E, EcoR V= Ec, Kpn I= K, Mlu I= M, Nde I= N, Nru I= Nr, Sac II= S, Sal I= Sa, Xho I= X, Xba I= Xb.
The 180 bp fragment was generated to give a Not I site upstream of the synthetic stop for construct excision. The fragment was cloned into the tTA or rtTA vectors using Pst I-Xho I cloning sites.

Step 5. An Xho I-EcoR I fragment was taken from the vector TNR1/CIV/S1 (Mohammed Nassar-UCL) containing the first 1.6 kb of the mouse NR1 subunit 5'UT region (up to the ATG of the first exon) and cloned upstream of the tTA/rtTA gene in the driver vectors.

Step 6. The rest of the available mouse NR1 5' UT region was inserted using a Sal I-Kpn I digested fragment cloned into Xho I-Kpn I sites in the driver vectors. The Xho I/Sal I site was destroyed by this manipulation. The overlap between the rest of the NR1-5'UT region and the 5'UT region from the TNR1/CIV/S1 vector was exploited so that the Kpn I site would be available for cloning.

The final construct (approximately 10 kb) was confirmed by restriction enzyme digestion and automated sequencing (Figure 3.26).
Figure 3.24. Diagram showing the assembly of steps 2, 3 and 4 involved in the construction of TDR-CSV-tTA and TDR-CSV-rtTA. Dashed lines represent insertion of fragments. Filled boxes and restriction enzyme symbols are according to the legend in Figure 3.23. Step 2, insertion of the tTA with the transcriptional stop into the multiple cloning site. Step 3, insertion of the rtTA gene before the transcriptional stop to make the rtTA version of the driver construct. Step 4, insertion of the 5' transcriptional stop before the tTA.
Figure 3.25. Diagram showing steps 5 and 6 for the construction of TDR-CVS-tTA. Dashed lines represent insertion of fragments. Filled boxes and restriction enzyme symbols are according to the legend in Figure 3.23. Step 5, insertion of a 1.5 kb 5'UT mNR1 region just 5' of tTA as an Xho I-EcoR I fragment. Step 6, insertion of the rest of the 5'UT mNR1 region as a 6.5 kb Sal I-Kpn I fragment.
Figure 3.26. Restriction enzyme map of the 10 kb 5'UT mNR1-tTA driver construct. Apa I= A, Bgl II= B, BamH I= Ba, Bsu36 I= Bs, Cla I= C, EcoR I= E, EcoR V= Ec, Hind III= H, Kpn I= K, Mlu I= M, Nde I= N, Not I= No, Nru I= Nr, Pst I= P, Sac II= S, Sal I= Sa, Xho I= X, Xba I= Xb.
3.6.3. Generation of 'driver mice' by pronuclear microinjection.

Several attempts were made by myself to produce transgenic mice by pronuclear microinjection, but these were unsuccessful. Although progeny were born from the oviduct transfer of injected eggs none of these mice were positive for the injected transgene (Table 8).

Further injections were made by Dr. Vasso Episkopou at the Hammersmith Hospital, London. From one injection session, thirty pups were born of which three were positive for the injected transgene. This was shown firstly by Southern blot and then confirmed by PCR genotyping. By using an EcoR V digest, the transgene could be cut at one position which would produce a single band the size of the injected transgene, if it has integrated into the genome as multiple copies in a head-to-tail array (Figure 3.27). A fragment from the tTA gene was used as the radiolabelled probe for the diagnostic Southern blot (see Figure 3.26 for location). Two of the founders (DRIV/F0/98-7002 and DRIV/F0/98-7003) contained the transgene at the expected size (10 kb) while one contained a single fragment slightly larger than the expected band at approximately 12 kb (DRIV/F0/98-7001), suggesting that the transgene had been inserted into the genome as a single copy (Figure 3.27). All three positive male founders are now being bred to generate F1 progeny in the C57/bl6 background for the analysis of transgene expression in the brain.
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Table 8. Attempts at pronuclear microinjection of the NR1-tTA driver construct (TDR-CVS-tTA) for the production of transgenic mice. Each row represents one injection session. The transgene was injected at concentrations of 1-5 ng/μl into C57/Bl6 single cell eggs. All pups produced were negative for the TDR-CVS-tTA transgene. Pups were weaned at 3 weeks of age.
Figure 3.27. Southern blot of DNA from the three NR1-αTA founders generated by pronuclear microinjection. DNA was digested with EcoR V and the blot was probed with a radiolabelled 750 bp EcoR I-Sac II fragment from the tTA gene. The positive control contained EcoR V digested wildtype mouse tail DNA spiked with 500 pg Not I digested TDR-CSV-αTA plasmid. The blot was exposed for 2 days at -80 °C.
4. Discussion.

4.1. Identification of residues on the NR2A subunit controlling glutamate potency in NR1/NR2A receptors.

These results have been previously discussed in Anson et al., (1998). This is particularly relevant for sections 4.1.3. and 4.1.4.

4.1.1. Glutamate potency is controlled by the NR2 subunits.

The agonist dose-response curves have shown that site-directed mutagenesis of NR2A residues reduced the potency of glutamate by up to three orders of magnitude with very little effect on the potency of glycine. This implies strongly, as explained in more detail later, that the binding site for glutamate on NMDA NR1/NR2A receptors is formed by the NR2A subunits. The residues found to be important in NR2A are fully conserved within the NR2 family (Figure 3.2), which suggests that in all types of NR1/NR2 NMDA receptors, glutamate effects are mediated by the NR2 subunit. For example this has been shown for the NR2D(T692A) mutation in NR1/NR2D receptors (see 4.2.1.). Comparison of this data with the effects of mutations at similar positions on the NR1 subunit (Hirai et al., 1996; Kuryatov et al., 1994; Wafford et al., 1995), imply that the glutamate effects mediated through the NR2 subunits and glycine effects mediated through the NR1 subunit, are each based on a common structural design.

While the experiments described here were well advanced, (Laube et al., 1997) reported that mutation of NR2B residues reduced glutamate potency on NR1/NR2B receptors. Three of their mutations were at equivalent positions to those found in this study; for two of them a comparable reduction in potency of glutamate was found (H460F and V660A) whereas for one (K459E) there was a considerably larger effect. Intriguingly, this residue is conserved in both NR2A and NR2B and may provide a difference between the glutamate binding pockets of NR2A and NR2B containing
receptors. At one position, NR2A S670, changing the serine to an alanine, S670A, produced a minimal effect on glutamate potency; the same serine to alanine mutation at the equivalent position on the NR1 subunit also had little effect on glycine potency (Kuryatov et al., 1994). Yet, (Laube et al., 1997) reported a reduction of glutamate potency by 180-fold for the mutation S664G in the NR2B subunit. The mutations that gave the three largest increases in glutamate EC$_{50}$ in Laube et al, (1997) (236-, 180-, and 118-fold) were all at different positions (E387A, K459E, and S664A respectively) from those that gave the largest shifts here (1027-, 321-, and 216-fold)(T671A, G669A, and H466A respectively).

Lummis et al., (1998) also presented results from NR2A mutations expressed with NR1 in HEK293 cells suggesting that the NR2A subunit controlled glutamate potency. Four of their mutations were at equivalent positions to those found in this study. The NR2A(V666A) mutation produced only a 4-fold reduction in glutamate potency (compared to a 10-fold shift found here), while the NR2A(G664A) mutation produced little change in glutamate potency. The NR2A(G669S) mutation (equivalent to our NR2A(G669A) mutation) reduced glutamate potency by more than 1000-fold (compared to 100-fold found here). Moreover, they also found little change in glutamate potency in the NR2A(K465D) mutation, which produced only a 10-fold reduction in glutamate potency, similar to the result found here (NR2A(K465E)). Thus the role of the lysine residue at position 465 (in terms of controlling glutamate potency) may be NR2B specific.

4.1.2. NR1 and NR2 subunits are both essential for the formation of a functional NMDA receptor.

The fact that glutamate and glycine potency is controlled by residues in the NR1 and NR2 subunits respectively (Anson et al., 1998; Kuryatov et al., 1994; Laube et al., 1997; Lummis et al., 1998) suggests that both subunits are probably necessary
for the formation of a functional NMDA receptor. This adds to the evidence that NMDA receptors are probably heteromeric.

As mentioned in the introduction, the NR1 cDNA was obtained by expression cloning in *Xenopus* oocytes gated by glutamate and glycine (Moriyoshi *et al*., 1991). Thus this led the suggestion that both glutamate and glycine binding sites exist on the NR1 subunit, having a role analogous to the α-subunit of the nicotinic acetylcholine receptor. However, 'homomeric' NR1 channels form relatively inefficiently compared to heteromeric NR1/NR2 channels. Furthermore, NR1 channels were found to have some properties similar to native NMDA receptors. But sensitivities to agonists and antagonists were quite different (see 1.2.6.), for instance by having a lower sensitivity to glutamate compared to NR1/NR2A-D heteromeric channels.

These NR1 only 'channels' also exhibit some unusual properties compared to heteromeric and native NMDA receptors. For example, NR1a receptor mediated currents are potentiated by Zn²⁺ at 10 μM concentrations (Hollmann *et al*., 1993). This contrasts with the observation that Zn²⁺ inhibits heteromeric NR1/NR2 receptors in a voltage- and voltage-independent manner.

'Homomeric' NR1 channels have only been observed in *Xenopus* oocytes but attempts to see 'NR1 only' currents in mammalian cell lines have been unsuccessful (Monyer *et al*., 1992). In fact it has been shown that neither NR1α or NR2α subunits are targeted to the cell surface efficiently unless they are coexpressed together in mammalian cell lines. NR1α subunits were found to be sequestered in the endoplasmic reticulum when expressed alone (McIlhinney *et al*., 1998). Thus most evidence suggests that it is unlikely that NR1 homomeric receptors exist *in vivo*.

So what could be responsible for this discrepancy in *Xenopus* oocytes? The oocytes may contain proteins essential for the assembly of functional recombinant NMDA receptors such as a chaperone protein allowing the NR1 subunit to reach the cell surface or an NR2-like subunit. Recent efforts have cloned two possible candidates from *Xenopus* frog brain, a non-NMDA like subunit (XenU1) and an NR1 like subunit (XenNR1) (Ishimaru *et al*., 1996; Soloviev *et al*., 1996).
The XenUl subunit has 36-40% sequence identity with rat non-NMDA receptors but did not form functional glutamate-gated channels when expressed alone in Xenopus oocytes. Another subunit, XenNR1 shares high amino acid sequence identity with the rat NR1 subunit with most of the differences in the N-terminal region. Coexpression of this subunit with XenU1 produced functional channels when expressed in HEK 293 cells. These channels were responsive to glutamate and NMDA in the presence of glycine, additionally they were also gated by AMPA and kainate but only in the presence of glycine.

It has been revealed that XenU1 mRNA is present (at low levels compared to brain) in Xenopus oocytes (Soloviev and Barnard, 1997). Therefore, is the interaction between XenU1 and the injected mammalian NR1 subunit responsible for 'NR1 homomeric' responses? And are the levels of XenU1 in oocytes high enough to form functional channels with NR1? Unfortunately glutamate evoked responses from the coexpression of mammalian NR1 and XenU1 in mammalian cell lines remain to be reported. It is possible that other unidentified proteins native to the oocyte may enhance the expression of NR1/XenU1 hybrid channels.

4.1.3. Evidence that reduced potency is caused by impaired binding of glutamate.

A reduction of potency is not necessarily caused by a change in the binding site, but can also be caused by a change of gating (Figure 4.1). The glutamate binding site might not be affected at all by the NR2A mutations and the entire effect could result from a reduction in the capability of the receptor to open the channel once the agonist is bound (gating) (Colquhoun, 1998). Additionally a binding assay for glutamate would not resolve this ambiguity because the radioligand would not discriminate between receptors in the AR (bound) and AR* (activated) states (Colquhoun and Farrant, 1993). In terms of binding, examination of receptors in the former state is important for determining amino acid residues involved in the agonist
binding process. In order to do this, it is necessary to show that the only the microscopic equilibrium binding constant of the initial binding step has been altered (Figure 4.1). This represents the binding of glutamate to the resting state of the receptor (i.e. before it changes to the active state/gating). Such a result would indicate that the mutated residues are involved in the binding of glutamate.

From the present data it is not possible to distinguish between residues that interact directly with the ligand and residues that shape the binding site without direct interaction with the ligand. This would require structural data of the binding pocket showing the positions of the mutated residues in relation to the ligand, although an attempt to do this has been made in Figure 4.4.
Figure 4.1. A simple mechanism describing the binding of an agonist and the subsequent activation of the receptor (gating step), based upon the two step mechanism proposed by del Castillo and Katz (1957).
The response to an agonist (A) is reflected by both binding and gating.

\[ R \rightleftharpoons AR \rightleftharpoons AR^* \]

binding  gating

Binding of a competitive antagonist (B) reflects only one step, and therefore tells us about the binding site.

\[ R \rightleftharpoons BR \]

binding

Figure 4.2. The components involved in the response of the receptor to an agonist and (lack of response) to a competitive antagonist.
The effect of these mutations on the binding site can be examined more clearly by using a competitive antagonist. Competitive antagonists, unlike agonists, are not expected to cause a change of the receptor conformation to higher-affinity active states (i.e. gating)(Figure 4.2). Thus any change in their binding is likely to result directly from a change in the binding site. It is conceivable that binding of the agonist might be altered in a mutant receptor without producing the same effect on the binding of a competitive antagonist because the binding pockets for the two compounds, although presumably overlapping, may not be identical. However, it was found that the T671A mutation increased the equilibrium binding constant, $K_B$ for APV by a factor of 255 compared with the wild-type. Therefore in the T671A mutant, APV binding is impaired greatly, which implies that its binding site, and therefore probably that for glutamate, has been altered by this mutation. A similar result was seen with the equilibrium binding constant for another competitive antagonist, R-CPP, which, for the T671A mutant, was reduced by 82-fold compared with wildtype receptors (see 3.3.2. and 4.4.).

The agonist dose-response curves also suggest a primary effect of the mutation on the binding site for glutamate. The main finding is that mutations in the NR2 subunit can produce an enormous reduction in the potency of glutamate (increase in its $EC_{50}$), with little change in the Hill slope of the equilibrium dose-response curve, and very little change in the potency of glycine. The three most effective mutants in this respect were H466A ($EC_{50}$ increased by a factor of 216, Hill slope, $n_H = 1.55 \pm 0.09$), G669A (factor of 321, $n_H = 1.38 \pm 0.07$) and T671A (factor of 1027, $n_H = 1.30 \pm 0.09$), and these can be compared with the wild-type for which the Hill slope was $n_H = 1.49 \pm 0.09$.

A reduction in the open-shut equilibrium constant (such that gating is less effective) could produce this result, and would not produce any detectable change in maximum response, as long as even the reduced value for the equilibrium constant is still such that most channels are open at equilibrium. What is the evidence for this not
happening? Firstly, for a wide range of co-operative mechanisms, we expect the shift of the \( EC_{50} \) to be proportional to the binding constants, but to be roughly proportional (in the case of two bindings being required) to the square root of the gating constant (as long as this equilibrium is well over towards the open state) (Colquhoun, 1998). In this case the 1000-fold increase in \( EC_{50} \) seen with T671A would require a roughly million-fold reduction in the gating constant. If this were to occur without a very drastic reduction of the maximum response, the gating constant for the wild-type channel would have to be enormously high. However, the available evidence suggests that it is \textit{not} enormously high. On the contrary, glutamate actually behaves like a partial agonist on the NMDA receptor, the maximum \( P_{\text{open}} \approx 0.3 \) (Gibb and Colquhoun, 1992). Thus in this situation a reduction in the gating constant would cause an enormous reduction in the maximum response. However, because of the uncertainties about the relative efficiency of expression, assembly and insertion of wild-type and mutant RNAs within the oocytes, it is difficult to determine whether maximum response has changed at all. Nevertheless there was no systematic difference detected between the maximum currents observed with mutant and wild-type receptors, so it is clear that changes by a factor of a million have not occurred (Table 3).

The other piece of supporting evidence comes from the observation that the Hill slope is little changed. Most kinetic mechanisms predict that the Hill slope would be reduced substantially if there were such a large effect on the gating constant as would be required to account for our observations, and no such reduction is seen (Colquhoun, 1998).

4.1.4. Interpretation of kinetic data of the T671A mutant-single channel data.

Initial single channel studies on NR1/NR2A(T671A) mutant receptors support the findings that binding rather than gating has been altered in this mutant (Anson \textit{et al.}
Single channel conductances of NR1/NR2A(T671A) channels were found to be similar to those evoked in the wildtype with both main and sublevels clearly seen (Figure 4.3). Additionally the transitional frequency of the T671A receptors between conductance levels was shown to be similar to that seen in wildtype recordings. Thus it seems that the active conformation of the mutant receptor has not been altered (nor the ability to switch between conductance states) and so this aspect of gating has not been changed in these receptors. However, kinetic differences were apparent between mutant and wild type receptors. For example the overall mean apparent length of the open periods was $4.33 \pm 0.71$ ms for T671A compared with $1.69 \pm 0.16$ ms for the wild type.

The observation that the mean apparent open period is longer for the T671A mutant could mean that the channels stay open for rather longer, which would imply some effect of the mutation on the shutting rate of the channel, i.e. on the gating process (Figure 4.1). This would suggest that some kinetic aspects of the receptor properties have been modified in this mutant and not only the initial binding step has been altered. Such an effect is inconsistent with the observed reduction in glutamate potency. Since such an effect on the shutting rate would cause a leftward shift in the glutamate dose-response curve (which is clearly not seen for T671A receptors). It is possible that such a change could be due to a more rapid dissociation of the agonist from the binding site (see below), rather than a change in gating, which would be expected to reduce the duration and/or frequency of brief shuttings such that fewer of them are detected so openings appear, incorrectly, to be longer.

Preliminary concentration jump experiments showed that the deactivation of T671A receptors after a 100 msec jump of 10 mM glutamate occurs with a time constant of $18.2 \pm 3.0$ ms (Anson et al., 1998), which is about 10 fold faster than that found for the deactivation of wild type NR1/NR2A channels after long pulses of glutamate (Monyer et al., 1992).

This decay rate is a reflection of the underlying channel activation. However, as the individual openings are longer in the mutant receptor, the fast decay cannot be
explained by shorter open times. Since the length of an activation depends on the number of times that the channel reopens while the agonist is still bound. The more rapid decay is likely to result from fewer reopensings during an activation for the mutant channel compared with the wild type. This could be due to either an increased rate of dissociation of glutamate or a reduced opening rate constant for the wild-type or the mutant channel. Since it seems that the wild-type and mutant receptors express with comparable efficiency, it is unlikely that the opening rate constant is greatly reduced in the mutant and thus it is unlikely to be the main reason for the faster decay. Therefore the main reason for the faster decay seems to be the faster dissociation of glutamate reducing the probability of the channel reopening.

This is consistent with a reduced affinity for glutamate.
Figure 4.3. Single channel currents recorded from outside-out patches from oocytes expressing NR1/NR2A(T671A) and wild-type NR1/NR2A recombinant receptors. Single channel measurements by L.C. Anson. Both main level (5 pS) and sublevel (4 pS) conductances can be seen in wildtype and mutant recordings. Figure from Anson et al., (1998).
4.1.5. Confirmation of these results by other structural models.

The recent publication of two papers have strengthened the findings by Laube et al., (1997) and the results presented here. Site-directed mutagenesis of amino acids in the expressed S1S2 domain of GluR4 revealed that R507 and E727 are involved in ligand (AMPA) binding while E424 is not (Lampinen et al., 1998). These residues are at the same positions as R493 in NR2B, D732 in NR1 and E387 in NR2B respectively (Laube et al., 1997; Williams et al., 1996).

The first structural model of an ionotropic glutamate receptor ligand binding site has revealed residues involved in agonist binding, confirming the structure-function data obtained here (Armstrong et al., 1998). A ligand binding domain from GluR2 was made containing only the S1 and S2 segments and from this a crystal structure of the ligand binding region was obtained in complex with kainate.

Amino acid residues at analogous positions to S664(NR2B), and D732(NR1) were found to contribute directly to the binding pocket of the receptor (at positions, S654 and E705 respectively in the GluR2 bi-lobar protein). Also the three amino acid residues (H466, G669 and T671) identified here as being likely candidates for ligand binding were found to be involved in receptor-ligand interactions in the binding pocket (at analogous positions in the GluR2 bi-lobar protein, Y450, G653 and T655 respectively). The authors suggested that the Y450 and T665 interacted with all ligands while the G653 residue interacted with 'specific ligands' (This was also suggested for the E402 residue, analogous to the E387(NR2B) mutation found by Laube et al., (1997)).

Figure 4.4. demonstrates the predicted location of mutated NR2A residues at corresponding positions within the GluR2 S1S2 protein ligand binding pocket. Based upon the crystal structure obtained by Armstrong et al., (1998) the H466, G669 and T671 residues are located close to kainate within the binding pocket. Whereas the G664 and T665 residues (which produced less than 5-fold reductions in glutamate
potency) are much further away from the ligand. Thus, in most cases the mutation of NR2A amino acid residues which produced large reductions in glutamate potency correlate with their proximity to the ligand within the binding pocket. The only exception is the S670 residue which is shown to be positioned very near to the ligand, but only produced a 2-fold shift in glutamate potency when mutated to an alanine. As mentioned beforehand, Laube et al., (1997) mutated the corresponding residue on the NR2B subunit to a glycine and observed an increase in glutamate $EC_{50}$ of more than 100-fold. The introduction of the glycine residue may have caused a structural change within the polypeptide backbone, disturbing the binding pocket. Thus the positioning of this serine residue rather than the contribution of the side-chain may be important for ligand interaction within the binding pocket.

The prediction of residues contributing to ligand binding made by other functional studies also agree well with the residues identified by this structural study. This is with the exception of two residues located in the S1 region which were both missed by functional studies. A proline residue in the GluR2 bi-lobar protein located at position 478 was identified as ligand binding but was not found by any of the previous NMDA receptor mutagenesis studies. This is also true for the nearby threonine residue at position 480 in the GluR2 protein. Both residues are highly conserved throughout the glutamate receptor family (Armstrong et al., 1998). In fact the corresponding proline residue (to P478 in GluR2) in the chick kainate binding protein produced little change in kainate binding when mutated (Paas et al., 1996).

Interestingly, the threonine residue which produced the biggest reduction in glutamate potency (T671 in NR2A, T655 in GluR2) is conserved throughout the glutamate receptor family. It is also conserved among the bacterial binding proteins from which the initial guesses for the location of the ligand binding site were made (Figure 4.5).
4.1.6. Future work.

Several areas of work remain to be investigated, firstly single channel analysis on the other mutants (H466A and G669A) would provide further insight into how these receptors have been changed in anyway at the single channel level.

Secondly work can focus on the differences in the results obtained between the NR2A and NR2B mutants. For example the K465E/K459E mutations in NR2A and NR2B respectively. Would a change of the lysine at this position to an alanine produce the same effect (change in side chain size rather than a change in charge)?

Additional point mutations could be made in the NR1 and NR2 subunits which correspond to the T480 and P478 residues in the GluR2 bi-lobar protein (T450 and P448 in the NR1 subunit and T494 and P492 in the NR2A subunit) identified as ligand binding by Armstrong et al., (1998). These residues are highly conserved in all glutamate receptor subunits and also in the bacterial binding proteins. Such mutations should produce large changes in glutamate/glycine potency.

Mapping of the antagonist binding site can be carried out by analysing the ten mutants made using the Schild method. It would be intriguing to see if any of the mutants produced large reductions in antagonist binding but not glutamate potency. Additionally, it would also be interesting to observe how the potency or pharmacological characteristics of other agonists such as NMDA are affected on the mutant receptors. Such studies may identify residues specifically interacting with NMDA, glutamate or APV.

Finally obtaining the crystal structure of the ligand binding pocket on the NMDA receptor in complex with both agonists (glutamate and glycine) and also for antagonists would confirm which residues interact with specific ligands vindicating/dispelling the predictions from functional studies. Comparison of these structures with those of AMPA or kainate receptor binding pockets would allow the specific residues involved in binding these agonists to be identified.
Figure 4.4. Location of mutated NR2A residues within the glutamate binding pocket (overleaf). Spacefilled representations of kainate and the residues corresponding to those mutated in the NR2A subunit are shown within the bilobar GluR2 S1S2 protein structure obtained by Armstrong et al., (1998). Corresponding residues in the GluR2 S1S2 protein were identified from the amino acid sequence alignment by Armstrong et al., (1998). The kainate molecule is coloured red and the position of the mutated residue is in cyan. The rest of the bilobar binding pocket is represented by a wireframe structure. The position of the residue within the GluR2 protein is given underneath with the corresponding residue position in the NR2A subunit in brackets. Structures were obtained using data from Armstrong et al., (1998) (pdb file accession code: 1gr2-Brookhaven National Laboratory) and visualised using the molecular visualisation software, RasMol v2.64 (Roger Sayle, 1994).
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<td>GluR5</td>
<td>653</td>
<td>GAV[boxed]RGSTMTFF</td>
</tr>
<tr>
<td>GluR6</td>
<td>652</td>
<td>GAVE[boxed]DGMTTF</td>
</tr>
<tr>
<td>NR1</td>
<td>664</td>
<td>ATV[boxed]KQSVDIYF</td>
</tr>
<tr>
<td>NR2A</td>
<td>664</td>
<td>GTP[boxed]NGSTERNI</td>
</tr>
<tr>
<td>LAOBP</td>
<td>114</td>
<td>VGVL[boxed]QGSTQEAY</td>
</tr>
<tr>
<td>HISJ</td>
<td>114</td>
<td>VGVL[boxed]QGTTQETF</td>
</tr>
</tbody>
</table>

**Figure 4.5.** Partial alignment of amino acid residues from glutamate receptor subunits and bacterial amino acid binding proteins in the S2 region showing common glycine and threonine residues. Glycine and threonine residues at positions analogous to NR2A(G669A) and NR2A(T671A) in the other proteins are boxed. Amino acid residues are numbered according to the predicted mature polypeptide.
4.1.7. General conclusions.

The arguments outlined above provide proof that the primary effect, at least for the three most effective mutations (H446A, G669A and T671A), is on the glutamate binding site and that both S1 and S2 regions contribute residues to it. The view of a conserved structural model of the agonist binding site is further supported by the identification of residues controlling ligand binding to chick cerebellar kainate binding protein (Paas et al., 1996) and bacterial expression of joined AMPA receptor S1 and S2 segments which reveal ligand binding (Kuusinen et al., 1995; Mori et al., 1992). A glycine-binding S1S2 domain formed from the NR1 subunit has already been created (Ivanovic et al., 1998) but this has yet to be shown for the NR2 S1S2 regions with glutamate.

This work strengthens the argument that NMDA receptors are heteromeric with glycine and glutamate binding sites located on the NR1 and NR2 subunits respectively. Additionally this suggests that NR1 homomeric responses observed in Xenopus oocytes are due to the interaction of the NR1 subunit with a yet to be found endogenous 'NR2-like subunit'. Non-NMDA (XenU1) and NMDA receptor (XenNR1) like subunits have been cloned from Xenopus laevis frogs, but whether these are the subunits responsible for NR1 'homomeric responses' seen in oocytes still remains to be proven (Soloviev and Barnard, 1997; Soloviev et al., 1996).

4.2. Introduction of point mutations into the NR2D subunit reduces glutamate potency in NR1/NR2D receptors.

4.2.1. The NR2D(T692A) mutation.

As expected, dose-response curves recorded from oocytes expressing NR1/NR2D(T692A) receptors produced a 1800-fold reduction in glutamate potency
compared to wild-type NR1/NR2D receptors. Additionally, this mutation produced little change in glycine potency or reduction in maximal current, suggesting that the primary effect has been on agonist binding rather than the gating of the receptor (see 4.1.3). This result is consistent with the effects of glutamate on the NR1/NR2A(T671A) receptors described earlier and supports the view that this threonine residue in the NR2 subunits has an important role in glutamate binding in NMDA receptors. As mentioned in 4.1.5, this threonine is conserved throughout the ionotropic glutamate receptor family, so its function must be crucial for the efficient formation of a glutamate binding site. Its importance for glutamate binding has been supported by the structural studies by Armstrong et al., (1998) and the structure-function studies by Paas et al., (1996) and Wo et al., (1999). Moreover, this mutation is useful for the kinetic study of NR1/NR2D containing receptors, for example is the long deactivation time associated with these receptors due to the length of time glutamate is bound to the NR1/NR2D receptor? As described earlier NR2D expression reaches its peak during early postnatal development and decreases after P12. Therefore, the long deactivation times associated with NR2D containing receptors may be necessary for the formation and strengthening of synaptic connections during this period (Dingledine et al., 1999; Wyllie et al., 1998).

4.2.2. The NR2D(K486E) mutation.

This mutation reduced glutamate potency by 34-fold compared to wild-type NR1/NR2D receptors, with little change in glycine potency. This reduction was smaller (10-fold) when the same mutation was expressed in NR1/NR2A receptors. However, when expressed in NR1/NR2B receptors, the reduction in glutamate potency was 180-fold compared to wild-type (Laube et al., 1997). Additionally, in NR1/NR2B receptors, this mutation produced an increase in APV and CPP IC₅₀. It is intriguing that the same conserved residue could exert such a different effect when inserted in the NR2B subunit.
4.3. Coexpression of NR1/NR2A/NR2A(T671A) cRNAs in oocytes-the stoichiometry question.

Expression of NR1/NR2A/NR2A(T671A) cRNAs in a ratio of 1:1:2, produced glutamate dose-response curves that could be clearly fitted with three components. These components had glutamate \( EC_{50} \)s that were similar to those of all wild-type NR1/NR2A and all NR1/NR2A(T671A) receptors and an intermediate \( EC_{50} \) that was more clearly defined when the ratio of NR2A:NR2A(T671A) was 2:1. This clear observation was probably due to the three-orders of magnitude difference between all wild-type and all mutant glutamate potencies. Expression of NR1/NR2B/NR2B(E387A) cRNAs showed a 'weakly triphasic' glutamate dose-response curve when the NR2B subunits were mixed in a 1:1 ratio (Laube et al., 1998). These authors exploited the two-orders of magnitude difference between the wild-type and mutant potencies and concluded that there was one intermediate channel species formed and thus two NR2 subunits within the receptor. Such a difference was probably not sufficient to clearly identify the intermediate within the dose-response curve. This approach requires the identification of clear phenotypes to distinguish them from all wild-type and all mutant receptor complexes and is also dependent upon a number of assumptions. Firstly, that the subunits assemble randomly, and do not effect the assembly of other subunits and that the subunits do not suffer from position dependent effects within the receptor complex (i.e. glutamate potency is not dependent on the position of the mutant within the receptor complex). Secondly, the mutation affects agonist binding and not gating and thirdly that each binding site behaves independently of the others.

In this study, one intermediate component was clearly observed in both 1:2:1 and 1:1:2 mixtures. The identification of one intermediate may suggest that there are three types of channel hetero-oligomers expressed and thus two NR2A subunits in the receptor complex, however there are a few issues with the data which may make the precise interpretation difficult. Firstly, the Hill slopes for a number of fits (Figures
3.17 and 3.21 for example), were much larger than the all wild-type and all mutant
dose-response curves (see Tables 6 and 7). This is probably due to the insufficient
number of points available for the data to be fit properly. A larger number of
concentration-data points would be needed to make the curve more precise (probably
at least 30-40 concentrations). Secondly, there is no maximal response that can be
measured for the mutant receptor (which can only be measured to 80 %), this made the
fitting more difficult. Although, such a mutation is better for identifying
'intermediates' than mutations which only cause a two-orders of magnitude reduction
of glutamate potency (i.e. used by Laube et al., (1998)). In the long run, the
NR2A(T671A) mutation maybe 'too extreme' for this purpose.

The more interesting question is whether the data can be fit by a four
component model, which would predict the existence of two intermediate components
within the dose-response curve (and thus imply the existence of three NR2 subunits
within the receptor). The 3- and 4-component constrained fits shown in Figure 3.21,
are virtually indistinguishable. In both cases, the curves do not fit the data points well
at the lower and higher ends of the curve when compared to the free fit shown in
Figure 3.18. The only way to distinguish between the fits would be to use a higher
precision to measure the dose-response curves, as explained earlier. But would the
resolution and precision of the data allow us to fit a four component curve anyway?
The range of concentrations measured may not allow us to observe a four component
fit and there is a possibility that two components may lie so close to each other that
they cannot be resolved.

It is plausible that two NR1 and two NR2 subunits form the NMDA receptor
complex. The voltage-gated K+ channel which shares several similar structural
features with glutamate receptors, is a tetramer, and it has been shown that two
glutamate and two glycine molecules are required to open the channel efficiently
(Benveniste and Mayer, 1991). A prokaryotic glutamate gated K+ selective channel
has been cloned recently (Chen et al., 1999) which shares amino acid homology with
both glutamate receptors and voltage-gated K+ channels (especially among the
glutamate binding and the pore-lining domains of these channels). This has been suggested as the 'missing link' between K⁺ channels and glutamate receptors and reinforces the argument that both channels are related. If the NMDA receptor was a pentamer and it would be assumed that each subunit bound either glutamate or glycine, what would be the role of the (presumed NR2) extra subunit? Could native NMDA receptors exist both as tetramers and pentamers? Are different NR1/NR2 receptor combinations more prone to a tetrameric assembly than a pentameric one and vice versa? Do recombinant expression systems force abnormal subunit ratios because of high expression levels? Structural studies of the receptor complex may be the only way to resolve this stoichiometric issue.

4.4. **R-CPP affinity is reduced in NR1/NR2A(T671A) receptors.**

The affinity of R-CPP for NR1/NR2A(T671A) receptors was reduced by 82-fold compared to wild-type NR1/NR2D receptors. This is 3-fold less than the reduction seen with APV (255-fold). A smaller shift in R-CPP than APV IC₅₀ was also observed by Laube et al., (1997) in one of their NR2B mutants. Thus for both competitive antagonists, the T671A mutation has changed their affinity and reinforces the idea that this residue is involved in glutamate binding. The difference in the reduction in affinity between the two antagonists suggests that residues involved in binding either APV or R-CPP or the way the antagonists interact with the binding site may be different for NR1/NR2A receptors.
4.5. Generation of an inducible 'tet-driver' mouse driven by the mouse NR1 5'UT region.

4.5.1. Advantages and disadvantages of the two component system.

Results have been reported of transgenic mice containing the tetracycline system (both driving and indicator components) integrated as a single transgene (Schultze et al., 1996). They observed induction ratios of 800-fold using the CMV promoter to drive tTA expression in certain tissues. Using the two component system in double transgenic mice they observed induction ratios of 5-240 fold.

Although this suggests that combination of the components in a single transgene is more effective than integrated as two separate transgenes, the two-component system of the tetracycline inducible gene expression system (involving a separate driver and effector) has several advantages for use in transgenic mice. Separate driver mice allow the tTA/rtTA system to be expressed in different tissues spatially, so that only the modified gene (effector) present in that area will be transcriptionally controlled by tetracycline. This 'mix and match' ability of driver and effector mice allows many genes to be examined under the control of different promoters. This saves time and effort in generating new lines of transgenic mice. A well characterised tissue-specific driver system established in transgenic mice would be of great use pin-pointing the actions of various genes in specific cell types.

Producing driver mice using pronuclear microinjection is advantageous because of the process of random integration. Expression of tTA/rtTA may be restricted to certain tissue types because of repressor elements in the surrounding locus of integration (Shockett and Schatz, 1996). This was found with the spatial expression of the indicator between different transgenic mice lines (Chen et al., 1998; Furth et al., 1994; Mansuy et al., 1998; Mayford et al., 1996; Schultze et al., 1996). However this may have been due to the CMV promoter in the driver. Random integration has been exploited to create a cre- transgenic mouse strain with ubiquitous expression (Schwenk et al., 1995). Various lines of transgenic driver mice can be
screened for spatial expression of the tTA/rtTA and any lines with obscure expression patterns can be exploited (e.g. if expression is localised in particular regions of the brain).

Variability in expression levels must be checked to ensure that lines of driver mice are expressing the tTA/rtTA to sufficient levels to control the effector. Copy numbers of driver and indicator constructs in mice lines may have to be matched, so that levels of tTA/rtTA are sufficient to control expression of the indicator. Otherwise poor transactivation may occur because of the unbalanced ratios. This is assuming no amplification occurs within the system when they are combined (one tTA/rtTA molecule may activate several indicator/effector components). Separating the components of the tetracycline system in different lines of mice allows control levels of indicator gene expression to be measured in the absence of any interference from transactivator activation. It would be useful to compare basal indicator levels between single and double transgenic mice in the presence of tetracycline, to examine how efficiently the system can reduce expression to basal levels. A comparison of expression levels would also indicate the 'leakiness' (tTA activity) of the system in the presence of tetracycline. Leakiness of the tet-minimal promoter in the presence of tetracycline may complicate the interpretation of data when examining driver/effector transgenic mice as levels of expression may be high enough to return a proportion of native function to the mouse. Thus it would be important to keep leakage to as low a level as possible.

Low expression levels due to position specific effects may be controlled by surrounding tetracycline system components with matrix attachment regions (MAR) to protect the transgene from the effects of regulatory elements flanking the integration site in the genome (McKnight et al., 1992). This would be used if expression levels of the tTA/rtTA were found to be too low for efficient functioning of the tet-system. The chicken lysozyme MAR has been used to insulate the whey acidic protein promoter from positional effects occurring to the transgene after integration (McKnight
1996). Although positional dependent repression was prevented, expression levels of
the transgene were found to be quite variable.

Creating separate driver and indicator/effector mice would require an extensive
breeding program and a large number of mice. Driver lines need to be crossed with
indicator/effector lines to generate double transgenics for the system to be examined at
all. Using another driver line would double the mice needed. Additionally with the
variability of transgene expression due to pronuclear microinjection many founder
mice may have to be screened for suitable expression levels before an ideal transgenic
line is established.

Another problem is genetic segregation of the components of the tet-system
during cross-breeding programs of driver and indicator/effector mice. Both
components may become localised in different areas of the genome which might
reduce the transactivation between the driver and indicator/effector components.
Progeny of double transgenic mice will have to be screened carefully to ensure that
efficiency of the system is similar through later generations of mice.

4.5.2. NR1 promoter driven transactivator expression.

Analysis of the NMDAR1 5' flanking region has shown that a 356bp region
(5'of exon 1 transcriptional start site) can drive reporter expression in rat PC12 cells
(Bai and Kusiak, 1995). A 3 kb fragment from this region has been characterised
containing one GSG and two SP1 motifs. No TATA box was found upstream of the
transcriptional start sites (Bai and Kusiak, 1993). It is hoped that the 8kb 5'
untranslated region fragment used to drive transactivator expression will be sufficient
to mimic the spatial and temporal expression of the native NR1 subunit. It should
have all the tissue-specific elements needed to direct expression in the brain. Large 5'
untranslated regions have been used successfully such as the 8.5 kb CaMkinase II
5'UT region (Mayford et al., 1996) in creating tTA/rtTA drivers.
4.5.3. Applications of inducible gene expression systems.

The tet-system exists as a dual system ideal for inducing and repressing gene expression. However the main concern is that leakiness (although low compared to other systems) of the tet-minimal promoter may be too high for efficient control of gene expression in the brain. It is likely that a degree of control will be obtained but a low level of expression will still occur in the presence/absence of tetracycline. The tTA system is more useful for fast repression because of the application of tetracycline while the rtTA is more applicable for inducing gene expression. The rtTA system still remains to be established in transgenic mice so it is not known whether it will perform as well as the tTA system. The tet-system is however more useful in studies of graded changes of gene expression and is ideal for experiments where control data is needed from the same animal. Application of tissue specific tTA and rtTA systems in the same animal would allow for selective expression of genes in different tissue types. Upon application of tetracycline, genes can be switched off while others can be turned on in the same animal. This would allow the study of the effect of certain genes in particular tissue types without the influence of other genes in other tissues.

For the complete inhibition of gene function the Cre-lox system would be more useful because the gene can be excised from the genome. However it is presently limited because of its lack of inducibility. Once inducible Cre-transgenic mice are established from the engineered embryonic stem cell line (Zhang et al., 1996) it would be more applicable in situations of studying gene function in animals by gene disruption but saving them from developmental problems encountered earlier.

4.5.4. Future work.

Once F1 progeny have been obtained from the three founders tTA expression of the three lines of mice containing the driver transgene can be examined by Northern blot analysis of brain mRNA. By crossing driver and indicator strains (where an
indicator gene is controlled by a tet-promoter) basal levels of indicator gene expression can be studied using luciferase or β-galactosidase histochemical assays in double transgenic mice. In this case drivers will be crossed with L7 luciferase indicator mice (Kistner et al., 1996). Additionally spatial and temporal expression of the indicator gene under the control of the driver can also be studied in this way. Induction ratios and expression levels of indicator genes can be examined in double transgenic mice under different concentrations and application periods of tetracycline/other analogues. Once the limits of the system have been established suitable drivers can be crossed with effector mice to study the effects of inducible gene expression in the brain.

4.5.5. Improvements in design.

Evidence from other attempts in making tTA driver mice have revealed several possible improvements in transgene design for the future.

So far successful attempts in creating functional tTA driver mice have involved inserting an artificial intron and splice sites at the 5' end (Mayford et al., 1996) or at the 3' end (Chen et al., 1998) of the tTA gene and the effector gene. Introns are thought to increase the levels of mRNA accumulation or transport efficiency from the nucleus to the cytoplasm (Chen et al., 1998). Secondly both cases used strong promoters which had been tested previously in transgenic mice.

Improvements in the tetracycline system have produced potentially useful variants of tTA. Newer forms of tTA have now become available with improved specificity for the tet-promoter and variable transactivator strength (Baron et al., 1997). This allows the transactivators to be matched with a promoter driving its expression of appropriate strength so that the correct combination can be found when integrated in the genome. Thus the activation potential of the transactivator can be adapted to the expression level of a specific promoter. This has potential uses for 'knock-in' strategies for cell-specific tTA expression.
4.5.6. General conclusions.

Two constructs have been made containing an 8 kb fragment of the 5'UT region from the mouse NR1 gene driving the expression of either the tTA/rtTA genes (driver). Only the tTA version has been used to generate transgenic mice by pronuclear microinjection and only three founders have been obtained. Further work is being carried out to analyse the levels and spatial expression of the tTA gene in these mice, either by looking at tTA expression alone or by crossing drivers with indicator mice and examining the expression of the indicator gene. The generation of different tissue-specific tTA driver mice will be of great use in controlling expression of targeted genes of interest. Hopefully one of the three driver lines obtained, will give sufficient levels of tTA expression for efficient control of gene expression in the brain.
5. References.


Laurie, D. J. and Seeburg, P. H. (1994b). Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. J-Neurosci 14, 3180-94.


