Metabotropic glutamate receptor mediated modulation of
N-methyl-d-aspartate (NMDA) receptor-channels in the rat hippocampus

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Abstract

*N*-methyl-d-aspartate receptors (NMDARs) were studied in granule cells of the rat hippocampus using high resolution patch-clamp electrophysiological techniques. The opening behaviour of the receptor-channel was observed in experiments employing the cell-attached configuration in order not to disturb the intracellular contents and maintain the normal physiology of the cell.

Co-activation of metabotropic glutamate receptors (mGluRs) located on the somatic membrane of these cells caused a robust and reproducible modulation in a number of the NMDARs single channel parameters. There was attenuation in the mean open time, burst length and cluster length of activation. Surprisingly there was also a decrease in the slope conductance of the receptor-channel.

This mGluR mediated modulation was occluded by applying cyclosporin A (CsA) which specifically inhibits the intracellular phosphatase 2B (calcineurin).

Whole-cell experiments employing the perforated patch configuration showed that mGluR activation could also attenuate whole-cell responses. However when the intracellular contents were disturbed either by using standard whole-cell techniques which cause washout of the cell or using fluoride (F\(^-\)) in the pipette to permeate the membrane and inhibit phosphatases, there was no predictable modulation of NMDA responses by mGluRs.

Voltage ramps performed during application of NMDA to these cells in the presence of varying concentrations of Mg\(^{2+}\) failed to show that this mGluR mediated modulation caused any change in the dissociation equilibrium constant for Mg\(^{2+}\) (\(K_{\text{Mg}}\)) or the fraction of the voltage field the blocker senses (\(\delta\)). This suggests that there is no change in Mg\(^{2+}\) sensitivity of the NMDA receptor-channel due to mGluR mediated modulation. Preliminary experiments testing the divalent permeability of the NMDAR-channel suggested there might be a decrease in the divalent/monovalent permeability ratio (\(P_{\text{Dz}}/P_{\text{M+}}\)).
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### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>1S, 3R ACPD</td>
<td>(1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>amino-3-hydroxy-5-methyl-4-isoxazole propionic acid</td>
</tr>
<tr>
<td>L-AP3</td>
<td>L(+)-2-amino-3-phosphonopropionic acid</td>
</tr>
<tr>
<td>Ba^{2+}</td>
<td>barium</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca^{2+}/calmodulin-dependant protein kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>CsF</td>
<td>caesium fluoride</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPSC</td>
<td>excitatory postsynaptic current</td>
</tr>
<tr>
<td>F^-</td>
<td>fluoride</td>
</tr>
<tr>
<td>GABA</td>
<td>y-amino-N-butyric acid</td>
</tr>
<tr>
<td>G-protein</td>
<td>GTP binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>IP_3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>K^+</td>
<td>potassium</td>
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<tr>
<td>KA</td>
<td>kainate</td>
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<tr>
<td>LTD</td>
<td>long term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>MCPG</td>
<td>(RS)-α-methyl-4-carboxyphenylglycine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>magnesium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>NMDA receptor</td>
</tr>
<tr>
<td>PI</td>
<td>phosphoinositol</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>zinc</td>
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Chapter 1 Introduction

The first observation that glutamate had an excitatory action on the central nervous system (CNS) was made by Hayashi in 1954. It was subsequently shown by Curtis & Watkins (1963) that ionophoretically applied glutamate was excitatory in many brain areas. Advances in the intervening forty years now point to glutamate as the principal mediator of fast excitatory synaptic transmission in the brain (reviewed by Watkins, 1994). Such an important function has made glutamate and in particular the receptors which mediate its actions an important subject for research and much is now known about the receptors' structure, function and involvement in physiological and pathological processes (for reviews see Wisden & Seeburg, 1993; Hollmann & Heinemann, 1994; Westbrook, 1994; M^Bain & Mayer, 1994).

Of the different sub-types of glutamate receptor, originally classified on the basis of their pharmacology, the N-methyl-D-aspartate receptor (NMDAR) is important in a number of cellular processes due to the high calcium (Ca^{2+}) permeability of the receptor's ion channel (Mayer & Westbrook, 1987; Forsythe & Westbrook, 1988; Ascher & Nowak, 1988; Schneggenburger et al., 1993; Schneggenburger, 1996). The receptor has proved to be of critical importance in processes which are influenced by the actions of intracellular Ca^{2+} such as neuronal development (Fox et al., 1992; Komuro & Rakic, 1993), neurotoxicity (Kaku et al., 1993; Rothman & Olney, 1995; Mody & MacDonald, 1995) and changes in synaptic efficacy (Bliss & Collingridge, 1993).
The main part of this thesis deals with the mechanisms involved in changes in NMDAR function following metabotropic glutamate receptor (mGluR) activation. Previous studies have shown that NMDAR function can be both attenuated (Colwell & Levine, 1994) or potentiated (Rahman & Neuman, 1996) by mGluRs in different brain areas. mGluRs activate GTP binding proteins (G-proteins) which regulate the enzymes phospholipase C (PLC) and adenylyl cyclase as well as directly affecting potassium (K⁺) channels (Huang et al., 1995; Wickman & Clapham, 1995; Schreibmayer et al., 1996) and Ca²⁺ channels (Dolphin, 1996; Herlitze et al., 1996). mGluRs may therefore influence other membrane proteins susceptible to modifications by enzymes such as kinases and phosphatases or which might be directly modulated by G-proteins.

Differences in NMDAR currents have also been observed in different configurations of the patch-clamp technique (Rosenmund et al., 1995; Clark et al., 1997). The basis of these discrepancies is thought to be due to the isolation of the receptor channel from its cellular environment when excising a membrane patch, leading to loss of the cellular mechanisms controlling these ion channels and so bringing about a change in their behaviour. The aims of this thesis are to quantify any changes in NMDAR function due to mGluR activation and to try to elucidate the mechanism of this modulation using pharmacological tools.
1.1 Glutamate receptors

Glutamate receptors have historically been categorised on the basis of pharmacological observations. Generally the ion channel receptors are split into three broad categories based on the affinity of the agonists NMDA, amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) for the receptors. Molecular techniques have shown that there are a profusion of different subunits of the different ionotropic receptors giving rise to the possibility of a great heterogeneity in native receptor structure and function (reviewed by Hollmann & Heinemann, 1994). A structurally and functionally distinct group, the metabotropic glutamate receptors (mGluR), are linked via G-proteins to intracellular effectors (reviewed by Pin & Duvoisin, 1995).

1.2 Ionotropic glutamate receptors

This thesis is concerned primarily with two types of receptor; the NMDAR and the mGluR. Therefore the introduction will deal primarily with these receptors. However as NMDAR structure can only be described in relation to what is known about non-NMDA glutamate receptors, these shall also be considered.

1.2.1 The NMDA receptor; nomenclature and subunit stoichiometry

Several subunits of the NMDAR have been cloned to date. These are further divided into two groups; the NR1 subunit (termed ζ1 for the receptor cloned from
mouse) (Moriyoshi et al., 1991; Yamazaki et al., 1992) is expressed ubiquitously throughout the brain whereas the four NR2 subunits; NR2A, NR2B, NR2C and NR2D (termed \(\epsilon_1-4\) respectively for the mouse), are expressed differentially and at different developmental stages (Meguro et al., 1992; Monyer et al., 1992; Monyer et al., 1994; Akazawa et al., 1994). Homomeric NR1 receptors expressed in *Xenopus* oocytes generate only small current responses (Moriyoshi et al., 1991). Functional recombinant receptors with properties similar to native receptors can only be formed by co-expressing the NR1 subunit with one or more of the NR2 subunits (Monyer et al., 1992; Kutsuwada et al., 1992; Stern et al., 1992); expression of NR2 subunits alone have been shown to be non-functional (Monyer et al., 1992). The specific pattern of expression in the CNS of NR2 subunits enables them to confer distinct functional properties on NMDARs in different brain regions (Farrant et al., 1994; Momiyama et al., 1996a; Piña-Crespo & Gibb, 1996). The precise subunit composition of native receptors is as yet unknown and it may be unreliable to take the stoichiometry of the nicotinic acetylcholine receptor (nAChR) which assembles as a pentamer of subunits (Anand et al., 1991; Cooper et al., 1991; Karlin, 1993) as an analogous model.

Functional studies indicate that receptors must assemble from at least two NR1 subunits (Béhé et al., 1995) which along with at least one copy of an NR2 subunit would suggest receptors containing at least a tri-mer of subunits. However biochemical data suggests a molecular mass of 730 kDa for the NMDAR complex consistent with at least two copies of the larger NR2 subunit (Brose et al., 1993) and this in addition to immuno-precipitation studies (Chazot et al., 1994) would
suggest that the receptor assembles, at the very least, as a tetramer of subunits. However current evidence from analysis of heteromeric recombinant mutants points to a pentamer of subunits forming the receptor (Béhé et al., 1995; Ferrer-Montiel & Montal, 1996).

In addition to the possible heterogeneity conferred on the NMDAR by the NR2 subunits, further potential diversity is possible due to the existence of several splice variants of the NR1 subunit (for review see Zukin & Bennett, 1995). Differences in the splice variants depend on three alternatively spliced exons. These encode a 21 amino acid sequence (N1) in the N-terminus domain and two 37 and 38 amino acid sequences (C1 and C2) adjacent to each other in the C-terminus domain. These insertions give eight possible splice forms of the NR1 subunit which have been shown by \textit{in situ} mRNA hybridisation studies to be differentially distributed in the CNS (Laurie & Seeburg, 1994) and which form receptors with altered pharmacological and physiological profiles (Anantharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Tingley et al., 1993; Ehlers et al., 1996).

1.2.2 Ionotropic glutamate receptor subunit transmembrane topology

Until recently the transmembrane topology of the ionotropic glutamate receptor subunits had been based on indirect evidence such as hydrophobicity measurements of the primary amino acid sequence (see Figure 1.1) and on the assumption that glutamate receptors were members of the same super-family of
Figure 1.1 shows a plot for hydrophobicity measurements of the amino acid sequence of the NR1 subunit of the NMDA receptor (created on ISREC home page*). The amino acid sequences corresponding to the areas previously thought to be the membrane spanning domains are marked 1-4. The lower part of the figure shows a diagrammatic representation of the proposed transmembrane topology of NMDA receptor subunits based on the model proposed for the GluR1 receptor (Hollmann et al., 1994). The three barrels represent the transmembrane domains previously referred to as TMD 1, 3, & 4. The area previously thought to be TMD 2 is shown to dip into the inner face of the membrane without traversing it. This region lines the channel pore and is analogous to the pore loops of voltage-gated potassium channels. The N-terminal is extracellular as is the large loop between the TMD 3 and 4. These two areas are thought to form the agonist binding domains. The C-terminal is intracellular and contains several consensus sites for protein phosphorylation (Tingley et al., 1993).

(*Location; http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html).
receptor proteins as the nAChRs (Betz, 1990). The commonly held interpretation of this data suggested a model of the receptor subunits with four transmembrane domains (TMD) and extracellular N- and C-termini. However several observations predicted this model to be flawed; in particular phosphorylation studies showed that the NR1 C-terminal must lie on the intracellular side of the membrane (Tingley et al., 1993).

The use of molecular genetic techniques has recently lead to a new proposal for the topology of these receptor subunits. Studies on the GluR1 AMPA receptor subunit (and a later study by Bennett & Dingledine (1995) on GluR3) suggest a topology of three TMDs with a fourth region, originally thought to be TM-II either lying on the inside of the membrane or anchored in the membrane without traversing it (Hollman, et al., 1994; Molnár, et al., 1994; Seal et al., 1995) (Figure 1.1). This region is homologous to the pore loops ("p-loops") of voltage gated K+ channels (Mackinnon, 1995). This gives a topological model of the subunit protein with an extracellular N-terminal and an intracellular C-terminal. Although NMDA and non-NMDA receptor subunits show less than 30% amino acid sequence homology (Hollman & Heinemann, 1994) it is quite probable that they share similar topologies and therefore it is a good hypothesis to take NMDAR subunit topology as being analogous to that shown for GluR1. Recent evidence for the NR1 subunit also goes some way to corroborating this evidence by showing the M3-M4 loop to be an extracellular domain forming part of the glycine binding site of the receptor (Hirai et al., 1996).
1.2.3 Physiological and pathological properties of the NMDA receptor

The NMDAR has been widely studied and has a number of specific differentiating features from the other ionotropic glutamate receptors. In addition to glutamate (or other structurally related agonists) binding as a requirement for channel gating, the NMDAR also requires glycine to bind as a co-agonist (Johnson & Ascher, 1987; Klechner & Dingledine, 1988) at a strychnine insensitive binding site separate but allosterically linked to the agonist binding site (Benveniste, et al., 1990).

Another unique feature of the receptor is due to the presence of a conserved asparagine residue in the “p-loop”-like region which confers a voltage dependent block by magnesium (Mg\(^{2+}\)) ions on NMDARs (Burnashev et al., 1992; Mori et al., 1992) which is eased on depolarisation of the cell (Mayer et al., 1984; Nowak et al., 1984). This provides a potential molecular basis for Hebbian processes (Hebb, 1949) which are thought to underlie learning and memory storage in the CNS (Cotman et al., 1988; Vianna Di Prisco, 1984; Rauschecker, 1991).

In addition, the receptor-channel can be influenced by a number of agents thought to have distinct binding sites on the receptor protein; polyamines (Scott et al., 1993; Forsythe, 1996; Johnson, 1996), zinc (Westbrook & Mayer 1987; Forsythe et al., 1988) & protons (Traynelis & Cull-Candy, 1990; 1991; Traynelis et al., 1995).
1.2.4 Single channel conductance of the NMDA receptor

Several studies on native NMDARs have shown that, taking into account the effect of calcium concentration ([Ca$^{2+}$]) on the single channel conductance (Ascher & Nowak, 1988), the receptors display heterogeneous conductance values. In addition to the generally accepted values of a main conductance level at 50pS (Cull-Candy & Ogden, 1985) with a sub-conductance level of 40pS in 1mM extracellular Ca$^{2+}$ (Gibb & Colquhoun, 1992) other studies have given a broader range of conductance levels (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Howe et al., 1991; Momiyama et al., 1996a; Piña-Crespo & Gibb, 1996). Presumably receptors in different brain areas and at different developmental stages have different subunit compositions (Monyer et al., 1994; Sheng et al., 1994) which seem to be responsible for the observed differences in conductances (Farrant et al., 1994; Piña-Crespo & Gibb, 1996; Momiyama et al., 1996a).

Expression of pairs of cloned subunits (NR1 in combination with each of the NR2D subunits) produces receptors with distinct conductances which can be directly compared to native receptors (Stern et al., 1992; Wyllie et al., 1996). These studies in combination with in situ hybridisation studies (Akazawa et al., 1994; Monyer et al., 1994) suggest that the “low-conductance” channels reported in immature Purkinje cells (Momiyama et al., 1996a) of the cerebellum and in immature granule cells of the hippocampus (Piña-Crespo & Gibb, 1996) contain the NR2D subunit of the receptor and, similarly, low-conductance channels
Table 1 summarises data from single channel studies which have given a range of conductance values from different cell types examined in both tissue culture and slice preparation and those of cloned receptors expressed in mammalian and non-mammalian cells. The low conductance channels thought to arise from combinations of subunits containing NR2C or NR2D have been described for both recombinant receptors (Stern et al., 1992; Wyllie et al., 1996) and native receptors (Farrant et al., 1994; Momiyama et al., 1996a; Piña-Crespo & Gibb, 1996). Ca\(^{2+}\) concentrations are given as total (HEPES buffered) or free (HCO\(_3\) buffered).

<table>
<thead>
<tr>
<th>Authors</th>
<th>Preparation</th>
<th>Conductance levels (pS) (n=x)</th>
<th>Calcium concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cull-Candy &amp; Usowicz, 1987</td>
<td>Large cerebellar neurons (cultures):</td>
<td>48±0.7 (23) / 38±0.8 (12) / 28±1.4 (7) / 18±0.5 (13) / 8.3±0.6 (17)</td>
<td>1mM</td>
</tr>
<tr>
<td>Howe et al., 1991</td>
<td>Cerebellar granule cells (cultures):</td>
<td>51.0±0.7 / 42.1±0.7 / 33.3±1.0 / 18.8±1.1 / 9.2±0.5 (11-30)</td>
<td>1mM</td>
</tr>
<tr>
<td>Gibb &amp; Colquhoun, 1992</td>
<td>Dissociated CA1 neurones:</td>
<td>51±1.5 / 37±1.5 (7)</td>
<td>1mM (0.84mM free)</td>
</tr>
<tr>
<td>Stern et al., 1992</td>
<td>Cloned receptors NR1-NR2A: NR1-NR2B: NR1-NR2C:</td>
<td>50.1±1.4 / 38.3±1.3 (6) / 50.9±0.4 / 38.7±1.5 (6) / 35.9±0.8 / 19.2±0.8 (4)</td>
<td>1mM (0.84mM free)</td>
</tr>
<tr>
<td>Farrant et al., 1994</td>
<td>Granule cells (cerebellar slices):</td>
<td>49.9±0.5 / 40±0.4 (21) / 33.0±2.3 / 20.3±2.1 (4)</td>
<td>1mM (0.84mM free)</td>
</tr>
<tr>
<td>Piña-Crespo &amp; Gibb, 1996</td>
<td>DG cells: Striatal neurones: (hippocampal slices)</td>
<td>48±2 / 36±1 (4) / 48±0.6 / 38±0.3 / 29±0.9 / 16±0.3 (3) / 50±1 / 38±1 (10)</td>
<td>1mM (0.84mM free)</td>
</tr>
<tr>
<td>Momiyama et al., 1996a</td>
<td>Purkinje cells (cerebellar slices):</td>
<td>37.2±0.6 / 17.6±0.6 (15)</td>
<td>1mM (0.84mM free)</td>
</tr>
<tr>
<td>Wyllie et al., 1996</td>
<td>Cloned receptors NR1-NR2D:</td>
<td>35±2 / 17±2 (5)</td>
<td>0.85mM</td>
</tr>
<tr>
<td>Clark et al., 1997</td>
<td>Granule cells from cerebellar slices: cell-attached: outside-out:</td>
<td>58.1±2.1 (4) / 59.0±1.3 (5) / 50.2±0.8 (6)</td>
<td>1mM (0.84mM free)</td>
</tr>
</tbody>
</table>
reported in the internal granule cell layer of the cerebellum (Farrant et al., 1994) arise from receptors containing the NR2C subunit of the receptor.

1.2.5 NMDA receptors’ contribution to synaptic currents

Excitatory postsynaptic currents (EPSCs) show two components; a fast component which is mediated by non-NMDARs and a slow time-course NMDAR mediated component which shows a slow rise-time (Hestrin et al., 1990) and a slow decay (Forsythe & Westbrook, 1988). Lester et al. (1990) showed that the slow decay of the NMDA mediated component is not due to repeated binding of glutamate to the receptor and suggested that it was due to the activation kinetics of the receptor. Using single channel studies Gibb & Colquhoun, (1991; 1992) showed that the activation of the NMDAR occurs in bursts grouped together in clusters and super-clusters. The authors suggested that the duration of super-clusters might be responsible for the slow decay of the synaptic current (Gibb & Colquhoun, 1991). However Edmonds & Colquhoun (1992) showed an obvious discrepancy between the length of receptor activations (recorded at very low agonist concentrations) and the decay of synaptic currents. This study showed that in addition to the behaviour of the channels underlying the EPSC the decay of the current is probably also characterised by the long latency between binding of agonist and the first opening of the channel (the 1st latency). Subsequently, indirect estimates of the NMDAR 1st latency in cultured hippocampal cells have been obtained using the channel blockers 9-aminoacridine (Benveniste & Mayer, 1995) and MK801 (Dzubay & Jahr, 1996).
1.2.6 Phosphorylation of the NMDA receptor

Many different membrane proteins can be phosphorylated by a number of intracellular kinases (Greengard, 1978; Swope et al., 1992; Moss et al., 1995). Phosphorylation occurs on transfer of a highly charged phosphate molecule to the hydroxyl groups of serine, threonine, or tyrosine residues on the substrate protein. This reaction is reversed by a hydrolysis reaction catalysed by protein phosphatases; the protein probably undergoes a conformation change which affects its function. This general method of controlling protein function is an attractive mechanism to explain modulation of neurotransmitter receptors. Indeed there is a wealth of information now accumulating about phosphorylation mechanisms influencing both pre-synaptic transmitter release mechanism (Malenka et al., 1986; Greengard et al., 1993; Südhof, 1995) and postsynaptic receptors (Markram & Segal, 1992; Blackstone et al., 1994; Moss et al., 1995; Tong et al., 1995).

1.2.7 Functional consequences of NMDA receptor phosphorylation

Table 2 summarises the results from a number of studies that have shown direct functional changes in NMDAR activation as a result of using inhibitors or activators of protein kinases and phosphatases (Mori et al., 1993; Lieberman & Mody, 1994; Wang et al., 1994; Wagner & Leonard, 1996) or by the direct intracellular application of kinases and phosphatases themselves (Chen & Huang, 1992; Wang & Salter, 1994). One study using single channel recording has
examined the effect of inhibiting phosphatases that might de-phosphorylate the NMDAR-channel complex and found increases in the channel open time, burst length & cluster length (Lieberman & Mody, 1994). In another study whole-cell responses to NMDA were shown to be potentiated by inhibition of protein tyrosine phosphatases (Wang & Salter, 1994).

<table>
<thead>
<tr>
<th>Authors</th>
<th>Preparation</th>
<th>Phosphatase involved</th>
<th>Effect on NMDA response</th>
<th>Protein Kinase involved</th>
<th>Effect on NMDA response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Markram &amp; Segal, 1991</td>
<td>CA1 neurones</td>
<td></td>
<td></td>
<td>Protein kinase C</td>
<td>PKC ( \uparrow )current</td>
</tr>
<tr>
<td>Urushihara et al., 1992</td>
<td>Whole rat brain mRNA (Xenopus)</td>
<td></td>
<td></td>
<td>Protein kinase C</td>
<td>Activation of PKC ( \uparrow )current</td>
</tr>
<tr>
<td>Chen &amp; Huang, 1992</td>
<td>Trigeminal neurones</td>
<td></td>
<td></td>
<td>Protein kinase C</td>
<td>PKC ( \uparrow )current reduction in Mg(^{2+}) block</td>
</tr>
<tr>
<td>Mori et al., 1993</td>
<td>ε1-ζ1 &amp; ε2-ζ1 (Xenopus)</td>
<td></td>
<td></td>
<td>Protein kinase C</td>
<td>Activation of PKC ( \uparrow )current</td>
</tr>
<tr>
<td>Lieberman &amp; Mody, 1994</td>
<td>Hippocampal granule cells</td>
<td>Calcineurin</td>
<td>Inhibition of phosphatase ( \uparrow )current</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang et al., 1994</td>
<td>Hippocampal neurones</td>
<td>Phosphatases 1 &amp; 2A</td>
<td>Inhibition of phosphatase ( \uparrow )current / phosphatase application ( \uparrow )current</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wang &amp; Salter, 1994</td>
<td>Spinal dorsal horn neurones</td>
<td>Tyrosine phosphatase</td>
<td>Inhibition of phosphatase ( \uparrow )current</td>
<td>Tyrosine kinase</td>
<td>Inhibition of kinase ( \uparrow )current / kinase application ( \uparrow )current</td>
</tr>
<tr>
<td>Wagner &amp; Leonard, 1996</td>
<td></td>
<td></td>
<td></td>
<td>Protein kinase C</td>
<td>PKC ( \uparrow )current no effect on Mg(^{2+}) block</td>
</tr>
<tr>
<td>Kohr &amp; Seeburg, 1996</td>
<td>NR1-NR2A (HEK)</td>
<td>src &amp; fyn kinases</td>
<td></td>
<td></td>
<td>Kinases ( \uparrow )current</td>
</tr>
</tbody>
</table>

Table 2 summarises several studies which have shown functional changes in NMDARs due to phosphatase or kinase activity. In general these studies suggest that phosphorylation of the NMDAR by kinase activity will potentiate NMDA responses, whereas dephosphorylation of the receptor due to phosphatase activity will attenuate the NMDAR mediated current.
A great deal of attention has focused on protein kinase C (PKC) as a modulator of NMDA mediated responses due to its abundance in the CNS (Brandt et al., 1987) and also its ability to readily phosphorylate other ion channels (Ross et al., 1988; Shearman et al., 1988; Browning et al., 1990; Gereau & Heinemann, 1996). A number of groups (see Table 2) have suggested that PKC can potentiate NMDA mediated currents. Chen & Huang (1992) showed that PKC modulated the NMDA receptor channel in dissociated trigeminal neurones by reducing the voltage dependant Mg^{2+} block. However Wagner & Leonard (1996) were unable to account for the large potentiation in current by a change in Mg^{2+} sensitivity of cloned receptors expressed in *Xenopus* oocytes. In general it is accepted that there is an overall potentiation of NMDA mediated responses by kinases and an overall attenuation of responses due to the action of phosphatases and it is suggested that these changes can be explained by alterations in the NMDARs single channel properties (Chen & Huang, 1992; Lieberman & Mody, 1994).

1.3 Involvement of NMDA receptors in synaptic plasticity

Bliss & Lømo (1973) and Bliss & Gardner-Medwin (1973) first described the phenomenon of a long lasting enhancement in synaptic strength after brief tetanic stimuli at synapses made between perforant path fibres and granule cells in the dentate gyrus of the hippocampus. Since then there has been an intense interest in this field as it is generally assumed that changes in synaptic strength are the physical mechanism underlying learning and memory formation (for reviews see Bliss & Collingridge, 1993; Bear & Malenka, 1994; Nicoll & Malenka, 1995).
great deal of subsequent information has demonstrated changes in synaptic strength (both potentiation and depression; long lasting and short term) (Artola & Singer, 1993) occurring at other synapses in the hippocampus and elsewhere in the CNS.

There are three basic properties which characterise classic long term potentiation (LTP); cooperativity, input-specificity and associativity (see Bliss & Collingridge, 1993). These properties combine to give Hebb-like characteristics to the induction of LTP; the synapse must be active when the postsynaptic cell is sufficiently depolarised for induction to occur. This requires a mechanism for detection of glutamate release and coincidental depolarisation of the postsynaptic neurone (Bourne & Nicoll, 1993). This is conveniently provided by the NMDAR; the physiological voltage dependant block by extracellular Mg$^{2+}$ is alleviated only when the postsynaptic membrane is depolarised due to activation of non-NMDA receptors and/or a decrease in inhibitory input (Davies et al., 1991). The permeability of the receptor-channel to Ca$^{2+}$ (Mayer & Westbrook, 1987) generates a rise in intracellular Ca$^{2+}$ (Regehr & Tank, 1990) causing activation of Ca$^{2+}$ sensitive intracellular signal transduction mechanisms (Mulkey et al., 1994) which convert the Ca$^{2+}$ transient into a persistent modification of synaptic transmission.

Collingridge et al. (1983) first demonstrated the critical role of NMDA receptors in the induction of LTP. Subsequent results have demonstrated the involvement of several different factors in LTP and long term depression (LTD) including mGluR
activation (Bashir et al., 1993), intracellular signalling cascades (Mulkey et al., 1994) and the recruitment of receptors at previously silent synapses (Isaac et al., 1995).

1.3.1 Phosphorylation mechanisms involved in LTP and LTD

A number of intracellular kinase and phosphatase enzymes are implicated in a cascade mechanism for the induction of LTP. In particular attention has focused on PKC’s ability to potentiate synaptic transmission in an LTP-like manner (Malenka et al., 1986; Malinow et al., 1989). However the role of PKC in LTP is not unequivocal (Muller et al., 1988) and other candidates have also been proposed as mediators of synaptic plasticity. It has been shown that inhibition of Ca^2+/calmodulin-dependant protein kinase II (CaMKII) prevents induction of LTP (Malinow et al., 1989) while protein tyrosine kinases are necessary for the induction but not the maintenance of LTP (O’Dell et al., 1991). CaMKII has been implicated in both LTP and LTD. It is suggested that phosphorylation of the CaMKII haloenzyme leads to it becoming constitutively active by a mechanism of Ca^{2+} independent autophosphorylation (Miller & Kennedy, 1986).

This array of different enzymes (kinases and phosphatases) involved in LTP and LTD has been brought together in a hypothesis proposed by Lisman (1994). This states that different enzyme pathways may be differentially activated by different stimuli to produce a cascade which either phosphorylates CaMKII in the case of LTP, or dephosphorylates it in the case of LTD. The compelling evidence for the
involvement of CaMKII in LTP and LTD fits well with the fact that it has been shown to be localised in high concentrations in the postsynaptic density (PSD) (reviewed by Kennedy, 1993). These mechanisms of generating long lasting changes in synaptic strength are by no means exclusively how memories are thought to be stored and new evidence, indicating that other mechanisms such as gene expression (Montminy, *et al.*, 1990; Schulman, H., 1995) may be involved in late stages to consolidate memories, is a whole new aspect of the subject.

1.3.2. Plasticity of the NMDA receptor-mediated component

Most of the work referred to above has dealt with synaptic changes of the AMPA receptor-mediated component of the EPSC. There is however evidence that NMDAR function may also be modulated at the synapse (Berretta *et al.*, 1991; Xie *et al.*, 1992; Bashir *et al.*, 1991; Kombian & Malenka, 1994; Tong *et al.*, 1995) giving rise to the possibility that there are mechanisms for modulating the degree of plasticity or the rigidity of the synapse. The assumption that NMDARs may be modulated in this way is reinforced by studies on extrasynaptic or cloned and expressed receptors mentioned in Section 1.2.6, which have shown direct modulation of the NMDAR properties by the action of kinases (Wagner & Leonard, 1996; Kohr & Seeburg, 1996) and phosphatases (Lieberman & Mody, 1994; Wang *et al.*, 1994).
1.4 Metabotropic glutamate receptors; nomenclature and transduction mechanisms

Until the mid-1980s the action of glutamate as a neurotransmitter was thought to be mediated exclusively via the gating of the ionotropic glutamate receptors discussed above. Metabotropic effects of glutamate were first described by Sladeczek et al. (1985) in cultured striatal neurones. This study in combination with other reports that glutamate could stimulate phospholipase C (Nicoletti et al., 1986, Sugiyama et al., 1987) suggested that glutamate acted on receptors coupled to G-proteins. The rapid progress in molecular genetic techniques in recent years has helped to explain these findings by, to date, characterising eight genes encoding more than eight different metabotropic glutamate receptors, due to the existence of splice variants.

The first receptor to be cloned was mGluR1 (Houamed et al., 1991; Masu et al., 1991) and subsequently this lead to the isolation of mGluR 2, 3, 4 (Tanabe et al., 1992), mGluR5 (Abe et al., 1992), mGluR6 (Nakajima, et al., 1993), mGluR7 (Okamoto, et al., 1994), mGluR8 (Duvoisin et al., 1995) & the splice variants of mGluR1 (Pin et al., 1992), mGluR4 (Simoncini et al., 1993) & mGluR5 (Minakami et al., 1993).

The mGluRs, in common with other G-protein linked receptors, have seven putative transmembrane regions, based on hydropathy measurements of amino acid residues and by analogy with the structure of bovine rhodopsin (Schertler et al.)
al., 1993). However they are much larger and have no sequence homology with the other seven transmembrane receptors forming a separate G-protein linked receptor family characterised by a large extracellular N-terminus.

It is not as yet categorically established which G-proteins are coupled to these receptors in neurones, however evidence from cloned receptors (Aramori & Nakanishi, 1992; Abe et al., 1992) points to differences in G-protein coupling between different receptors and also between different splice variants of the same receptor. Native receptors have been shown to be coupled to either G<sub>1</sub> - G<sub>0</sub> pertussis toxin (PTX) sensitive (Nicoletti et al., 1988) or G<sub>q</sub> PTX insensitive G-proteins (Sladeczek et al., 1985).

The mGluRs are classified into three groups based on their amino acid sequence homology. Receptors in each group also differ in the signal transduction mechanism they are linked to (reviewed by Pin & Duvoisin, 1995). mGluR1 and mGluR5 are both members of Group I; these receptors stimulate PLC and increase phosphoinositide (PI) turnover releasing Ca<sup>2+</sup> from intracellular stores.

mGluR2 and mGluR3 are Group II receptors and strongly inhibit forskolin stimulation of cyclic adenosine monophosphate (cAMP) production via a PTX sensitive G-protein. Group III consists of the remaining four receptors which are also negatively coupled to cAMP production but produce less inhibition than the Group II receptors.
1.4.1 Physiological roles for metabotropic glutamate receptors

The diversity in structure and transduction mechanisms of the mGluRs is reflected in their many proposed physiological functions. Forsythe & Clements (1990) showed the first clear demonstration of a presynaptic metabotropic effect. Since then several studies in the hippocampus have shown Group III receptors causing pre-synaptic depression of inhibitory (Liu et al., 1993; Desai et al., 1994) and excitatory synaptic transmission (Baskys & Malenka, 1991; Parcelli & Kelso, 1991; Desai et al., 1994). Group I mGluRs have also been implicated in presynaptic inhibition of glutamatergic & GABAergic transmission (Gereau & Conn, 1995). Taken together with information from the accessory olfactory bulb where presynaptic Group II receptors have been implicated in inhibition of GABAergic transmission (Hayashi et al., 1993) and at the calyx of Held (Forsythe & Barnes-Davies, 1993; Barnes-Davies & Forsythe, 1995) where one or more mGluRs fitting the pharmacological profile of mGluR subtypes 4, 6, 7, & 8 inhibit glutamatergic transmission by suppression of a presynaptic Ca\(^{2+}\) current (Takahashi et al., 1996), mGluRs from each Group seem to be important in presynaptic mechanisms of modulation.

mGluRs show differential expression in the CNS (Catania, et al., 1994). All the experiments in this thesis are in hippocampal slices and specifically in the dentate gyrus granule cells. Therefore this section will concentrate mainly on the mGluRs expressed in this region which are the Group I receptors; mGluR1 (Fotuhi et al.,
1993; Martin et al., 1992) & mGluR5 (Shigemoto et al., 1993) and also one of the Group II receptors, mGluR2 (Ohishi et al., 1993).

1.4.2 Metabotropic receptor phosphorylation

In common with ionotropic glutamate receptors there is increasing evidence that mGluR activity can be modulated by phosphorylation. mGluR mediated IP$_3$ hydrolysis has been shown to be inhibited by direct stimulation of PKC using phorbol esters (Canonico et al., 1988; Schoepp & Johnson, 1988). In addition the receptor has several serine and threonine residues on the intracellular C-terminal (Tanabe et al., 1992) which may provide consensus sites for phosphorylation. In common with β-adrenoceptors (Lefkowitz et al., 1990) it has recently been shown for mGluR5 that these phosphorylation sites are important in regulating receptor desensitisation (Gereau & Heinemann, 1996), intracellular Ca$^{2+}$ signalling (Kawabata et al., 1996) and could also possibly influence G-protein coupling. In addition mGluR1α also shows basal levels of phosphorylation and activation of the receptor by glutamate greatly increases this by a mechanism involving PKC (Alaluf et al., 1995).

1.4.3 Metabotropic receptor involvement in synaptic potentiation and depression

There has been a lot of contention as to the precise role of mGluRs in synaptic plasticity; currently evidence of their role is fragmentary although an increasing amount of evidence suggests that mGluRs are involved in certain forms of LTP.
and LTD (Nakanishi, 1994). The link between Group I receptors and PKC via the bifurcating diacylglycerol (DAG), inositol triphosphate (IP$_3$) pathway initially implicated these receptors in a postsynaptic model of LTP.

That mGluRs are involved in mossy fiber and CA1 LTP at all is still contentious. Evidence from some groups has shown that LTP is not affected by antagonism of mGluRs by (RS)-α-methyl-4-carboxyphenylglycine (MCPG) (Chinestra et al., 1993; Manzoni et al., 1994), a specific mGluR antagonist (Watkins & Collingridge, 1994). Evidence from gene knockout experiments has also shown that mossy fiber LTP generated in mutant mice lacking mGluR1 is identical to that in wild-type mice (Hsia et al., 1995).

Other groups have reported that mGluRs are involved in mossy fiber LTP although the mechanism of their contribution and whether they are essential to the process are still moot points (see Ben-Ari & Aniksztejn (1995) for review of two proposed models).

One proposed mechanism for the involvement of mGluRs in this form of LTP suggests that activation of postsynaptic mGluRs augments NMDA currents (O’Connor et al., 1994) which in turn are responsible for the induction of LTP of the AMPA mediated component (Anikstejn, et al., 1992; McGuinness et al., 1991).
The alternative model proposes that mGluRs act postsynaptically to directly modulate synaptic efficiency by increasing the AMPA mediated conductance (Bortolotto & Collingridge, 1993 & 1995). MCPG has been shown to prevent the induction of LTP in CA1 and CA3 regions of the hippocampus (Bashir et al., 1993). Additional corroborative reports suggest that, contrary to the findings of Hsia et al. (1995), mutant mice lacking mGluR1 show a marked lack of mossy fiber LTP (Aiba et al., 1994).

In addition to the controversy surrounding mGluR involvement in LTP, these receptors have also been implicated in LTD. LTD in the hippocampus is a less well characterised phenomenon than LTP. Contrary to LTP induction protocols, hippocampal LTD is induced by prolonged low frequency stimuli and leads to a weakening of excitatory synaptic transmission (Mulkey & Malenka, 1992; Dudek & Bear, 1992; Malenka, 1993). The best characterised form of LTD involving mGluRs occurs in the cerebellum at the parallel fiber-Purkinje cell synapse (for review see Linden & Connor, 1993; Linden, 1994). Antibody inactivation of mGluR1 blocks the induction of LTD suggesting activation of this receptor is a necessary requirement for LTD at this synapse (Shigemoto, et al., 1994).

In addition to the contradictory results from different groups concerning the role of mGluRs in hippocampal LTP, a further complication is that mGluR mediated LTD can also be generated in the hippocampus (O’Mara et al., 1995) at the same synapses where LTP has been exhaustively studied. However the contribution of mGluRs to LTD is not widely disputed as it is in LTP although other aspects of
this form of plasticity, such as the role of NMDARs, have yet to be resolved (Mulkey & Malenka, 1992; Dudek & Bear, 1992; Bolshakov & Siegelbaum, 1994; Bashir & Collingridge, 1994). It has been suggested that the apparent disparity caused by the ability of synapses to be potentiated or depressed may be explained by different intracellular processes being activated by different intracellular Ca$$^{2+}$$ transients (Lisman, 1994).

1.4.4 Interactions between metabotropic glutamate & NMDA receptors

Synaptic potentiation or depression are generally thought of as changes in the non-NMDA mediated current at the synapse whether due to pre- and/or post-synaptic mechanisms. However the NMDA mediated component of the synaptic current can also be potentiated (Berretta et al., 1991) or depressed (Xie et al., 1992; Selig et al., 1995). Similarly it is also now generally accepted that mGluR activation can modulate NMDA mediated synaptic currents either by potentiation (O’Connor et al., 1994) or by depression (Yi et al., 1995) of the current. Whether this modulation is due to a direct modification of postsynaptic NMDAR properties is still unknown.

It is therefore interesting to see if mGluRs might directly modulate NMDAR function. Kelso et al. (1992) showed that trans-ACPD potentiated NMDA responses in Xenopus oocytes via a mechanism involving PKC. Other evidence also suggests that mGluRs may enhance NMDA responses in acutely isolated dorsal horn neurons (Bleakman et al., 1992), neocortical neurones (Rahman &
Neuman, 1996), CA1 cells of the hippocampus (Harvey & Collingridge, 1993; Aniksztejn et al., 1992) and in visual cortex neurones (Wang & Daw, 1996).

However there is also evidence that mGluR activation can attenuate NMDAR mediated responses in neostriatum (Colwell & Levine, 1994) and cortical neurones (Koh et al., 1991; Birrell et al., 1993).

The discrepancies between these observations may be due to a number of reasons. Firstly, and most obviously, there are differences between cell types not only in mGluR expression but also in other factors which could contribute such as differences in intracellular mechanisms for Ca$^{2+}$ buffering or types and predominance of different kinases and phosphatases. Secondly methodological differences in observing the NMDAR mediated component also differed greatly and in most cases were not very high resolution.

1.5 Experiments undertaken in this thesis

This thesis aims to look at the modulation of native NMDARs by mGluRs. This is accomplished in Chapter 3 by using patch-clamp techniques to study NMDAR single channel openings in cell-attached membrane patches. Inhibition of intracellular enzymes is also used to further explore the mechanism of modulation. In Chapter 4 the modulation is investigated further by testing Mg$^{2+}$ sensitivity and divalent permeability of the NMDAR after mGluR modulation. The final
results chapter, Chapter 5, looks at whole-cell current modulation and how this is affected by a number of manipulations in the experimental protocol.
Chapter 2 Methods

NMDAR function was studied in a number of different configurations of the patch-clamp technique with the intention, in most cases, of causing as little disruption to the normal cell physiology.

2.1 Slice Preparation

Thin slices were prepared from 13-16 day old Sprague-Dawley rats as described previously by Edwards et al., (1989). Rats were killed by decapitation and the brain tissue was rapidly submerged in ice-cold oxygenated Krebs solution (see Section 2.2) containing 4mM Mg\(^{2+}\). One hemisphere of the brain was chosen and a flat surface cut along the top. The tissue was then glued on to the tissue block of a Campden Vibroslice with cyanoacrylate glue. 250\(\mu\)m thick horizontal slices were cut and the hippocampal formation dissected from the whole slice.

Hippocampal slices were transferred to a holding chamber and incubated at 32\(^\circ\)C for a minimum of 30 minutes before being transferred to a recording chamber mounted on the stage of the microscope (Zeiss Axioskop FS) and viewed at x640 magnification under Normarski optics. The microscope was mounted on a Photon Control (Cambridge, UK) anti-vibration air-damped flotation table. A CCD camera (Radio Spares) with contrast enhancing circuitry (E.R. Dyett, Department of Pharmacology, University College London) was used to view the slices on a monitor.
and visual assessment and surface cleaning of the cells in the dentate gyrus was done as described by Gibb & Edwards (1994).

2.2 Solutions

2.2.1 Standard Krebs solution

A standard Krebs solution composed of (in mM): glucose 25; NaCl 125; KCl 2.4; NaH₂PO₄ 1.2; NaHCO₃ 25; CaCl₂ 1 MgCl₂ 4; bubbled with 5% / 95% CO₂ / O₂, was used to prepare and incubate the slices. All salts were purchased from BDH and gases from BOC. A similar solution but containing no MgCl₂ was used to continuously perfuse the slices in the recording chamber. CaCl₂ was substituted by BaCl₂ in the extracellular solutions used for some of the perforated patch whole-cell experiments and in some early whole-cell experiments Krebs solution containing 2mM CaCl₂ was used.

2.2.2 Patch-clamp pipette solutions

The pipette solution for cell-attached recording was prepared with Aristar (BDH) high purity chemicals to minimise divalent contamination. The composition of this pipette solution was (in mM) HEPES 10; NaCl 140; CaCl₂ 1. pH was adjusted to 7.4 with 1M NaOH. HEPES was Ultrol® grade purchased from Calbiochem.
The solution used for calcium free cell-attached recording was composed of (in mM) CsF 120; CsCl 10; EGTA 10; HEPES 10. pH was corrected to 7.4 with 1M CsOH. CsF and CsCl were purchased from Fluka and EGTA and gluconolactone were both purchased from Sigma.

The composition of the sodium gluconate pipette solution used for whole-cell and perforated patch experiments was (in mM) gluconolactone 140; NaOH 140; HEPES 10; EGTA 11; NaCl 10; adjusted to pH 7.4 with 1M NaOH. The solution needed to be left for 3-4 hours at room temperature for the pH to equilibrate after adding NaOH. It was estimated that between 2.8ml and 3ml of 1mM NaOH was required per 100ml of solution to adjust to the required pH. This increased the final [Na+] from 150mM to between 178-180mM.

In some of the perforated patch whole-cell experiments a CsF pipette solution was used whose composition was (in mM) CsF 110; CsCl 30; NaCl 4; CaCl2 0.5; EGTA 5; HEPES 10; pH adjusted to 7.4 using CsOH.

2.2.3 Chemicals and compounds

NMDA, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid {(1S,3R)-ACPD}, (S)-α-Amino-4-bromo-3-hydroxyl-5-isoxazolepropionic acid (AMPA) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3) were supplied by Tocris Cookson (Bristol, UK); bicuculline and tetrodotoxin (TTX) were supplied by Sigma; calphostin C was supplied by Calbiochem. 200nM calphostin C containing Krebs
was freshly prepared for each experiment from 300µl aliquots containing 10µM calphostin C in normal Krebs which were frozen and protected from light. Cyclosporin A (CsA) was supplied by Sandoz. Krebs containing 20-100nM cyclosporin A was freshly prepared for each experiment from a 100µM stock solution. All solutions containing cyclosporin were made up and stored in plastic containers as cyclosporin sticks readily to glass. L-glutamate and glycine were purchased from Fluka.

2.2.4 Preparation of pipette solution for perforated patch recording

Gluconate solution containing amphotericin B was prepared by sonicating (Heat Systems Ultrasonic Processor XL) 1.5mg of amphotericin B (Sigma) in 20µl of DMSO (dimethyl sulfoxide) (Sigma) to produce a stock solution which had a very dark yellow/brown colour. 2µl of this stock solution was added to 1ml of the gluconate pipette solution and again sonicated producing a suspension with a light yellow-milky colour. This solution was kept on ice, shielded from light during the course of the experiment and needed to be freshly prepared every two hours as solution activity began to dissipate at times beyond this.

2.3 Recording configurations

In this study patch-clamp recordings were made from the somatic membranes of granule cells in rat hippocampal slices (Edwards et al., 1989; Gibb & Edwards, 1994, Gibb, 1995) using an Axon instruments (CA, USA) Axopatch 200A amplifier.
Recordings were monitored during the experiment using a HAMEG digital oscilloscope (signals were filtered for viewing at 2kHz) and recorded (gain of x100 and a combined bandwidth of the amplifier and DAT recorder of ~7kHz) onto digital audio tape (Intracell DT1202, Cambridge UK) for off-line analysis.

2.3.1 Cell-attached patch clamp recordings

Patch electrodes were manufactured from thick-walled borosilicate glass (Clark Electromedical GC150F 7.5) using an upright, two-stage puller (List Medical L/M-3P-A). Stray capacitance arising from contact between the glass wall of the pipette and the solution in the bath was reduced by coating pipettes with Sylgard® resin (Dow Corning) to within 100µm of their tips. Pipettes were mounted in the patch-clamp headstage (Axopatch 200A) holder and a small amount of positive pressure applied before pushing the pipette tip close to the cleaned surface of a granule cell using a coarse 3-way manual translator (Photon Control) and a fine piezo manipulator (Photon Control) driven by an MD3-75C piezo-controller (Photon Control).

The positive pressure was released and a small amount of suction applied to form the seal (see Figure 2.1). To increase the seal formation success rate, pipettes were fire polished on a Narashige MF-83 microforge to give a final tip resistance of 10-15 MΩ. In the majority of experiments 5µM NMDA was included in the pipette solution along with 5µM glycine. However in some earlier experiments 10µM glutamate was also used as the agonist. Double openings, which can be a problem
Figure 2.1 shows a cartoon representation of the cell attached recording configuration. Recordings were made from granule cells of the dentate gyrus (DG) from 250 μm hippocampal slices. Slices were immobilised in a bath using a nylon mesh and constantly perfused with a standard Krebs solution (see section 2.6) bubbled with 5% / 95% CO₂ / O₂. Visual selection and cleaning of cells in the DG was performed before patch formation and recording. Agonist was included in the patch pipette to evoke channel openings.
during analysis and are commonly found in the outside-out configuration even at low agonist concentrations, were surprisingly rare in the cell attached mode.

One to two minute recordings were made at each of several pipette potentials so that plots of single channel current against pipette potential could be made. In the experiments involving activation of mGlurRs, 50μM of the specific agonist (1S, 3R)-1-aminocyclopentane-1,3-dicaboxylc acid (1S, 3R-ACPD) was applied to the slice in the perfusate for a minimum of ten minutes and steady state recordings were again made at several pipette potentials in the continued presence of ACPD.

In two cells after control recordings had been made, 20-100nM of the selective phosphatase 2B (calcineurin) inhibitor cyclosporin A (CsA) was applied to the slice, in the perfusate, for a minimum of 20 minutes prior to application of ACPD and then recording was repeated using the above protocol. These recordings were technically difficult as stable recordings were required to last over a period greater than one hour.

2.3.2 Inside-out patches and patch cramming

Inside-out patches were formed by first making cell-attached patches then excising the patch from the cell membrane by a quick movement of the coarse manipulator to form an inside-out patch. Occasionally a vesicle seemed to form in the pipette tip and could be remedied with varying degrees of success by quickly moving the patch across the liquid-air interface of the bath.
In one group of experiments patches were crammed (Kramer, 1990) back into the parent cell by moving the excised inside-out patch back towards the cell and then using the piezo driven micro-manipulator to push the patch through the membrane into the cell.

2.3.3 Outside-out patches and perforated vesicles

The outside-out configuration of the patch-clamp technique was adopted in some experiments. Sylgard coated pipettes were back-filled with sodium gluconate intracellular solution (Section 2.2) and, whilst applying positive pressure, brought close to the cell chosen to be patched. On formation of a giga-ohm seal, suction was applied to break through the cell membrane and the pipette was slowly moved away from the cell using the piezo-driven micro-manipulator to form an outside-out patch. Extracellular solution containing low concentrations of the agonist NMDA or glutamate along with glycine were bath applied to evoke channel openings.

Another related configuration adopted in some experiments involved the formation of the whole-cell perforated patch configuration using amphotericin B as in Section 2.3.4. The electrode was then withdrawn slowly, as described above, to form a perforated vesicle (Levitan & Kramer, 1990). This configuration possibly allows retention of some of the intracellular species in the vesicle which are lost when making outside-out patches.
2.3.4 Whole-cell and whole-cell perforated patch recording

Whole-cell recordings were made from dentate gyrus granule cells using a sodium gluconate pipette solution. Pipettes were pulled from borosilicate glass as before and fire polished to give a final tip resistance of approximately 10 MΩ. Positive pressure was applied to the pipette and the tip of the pipette was pushed against the cell membrane. The positive pressure was removed and a seal allowed to form assisted by applying a little suction. The cell membrane was then disrupted using short pulses of strong suction to form the whole-cell configuration.

Whole-cell perforated patch recordings were made using methods adapted from Horn & Marty (1988) and Rae et al. (1991). This technique employs the use of macrolide antibiotics in the patch pipette to form pores in the cell membrane which allow small monovalent ions such as Na⁺, K⁺ and Cl⁻ to pass but exclude larger divalent ions such as Ca²⁺ allowing us to record from the cell without disrupting the intracellular contents to any great extent.

The tip of the pipette was dipped into a sodium gluconate pipette solution for 35 seconds and then the pipette was back-filled with a freshly prepared amphotericin B containing solution (Section 2.2.4). The pipette was brought close to the cell without using any positive pressure and seals were generally made by applying gentle suction. This method gave series resistances in the order of 30-60MΩ within 5 minutes of obtaining the seal. Capacitance transients were corrected for by the compensation circuitry on the Axopatch 200A. Although series resistances
attainable with this method are high compared to those using conventional whole-cell techniques, due to the high input resistance and small size of the NMDA response from these cells, there is little problem with voltage control.

In the earlier experiments when generally only whole-cell recordings were made, agonists were applied using a flow pipe situated ~100μm from the cell soma. In the later experiments when the perforated patch technique was used agonists were applied in the bathing solution. The protocol adopted for the experiments in which voltage ramps were performed involved three agonist applications (20μM NMDA and 5μM glycine) followed by application of 50μM ACPD for a minimum of 10 minutes and then a further three agonist applications (in the continuous presence of ACPD). In some cases the slice was then again bathed in the control solution for a ten minute period, and a further three agonist applications were made.

2.4 Analysis of single channel records

2.4.1 Measurement of channel amplitudes and dwell times

Recordings were amplified and filtered at 1-2 kHz (8 pole Bessel) and continuously sampled at 10-20 kHz onto an IBM compatible PC (Dell) using a Cambridge Electronic Design 1401 plus interface. Single channel records were analysed using SCAN, a time course fitting program (Colquhoun & Sigworth, 1995) which fit the scaled step response of the recording system to closed-open, open-open and open-closed transitions in the data record. This fitting procedure allows the time point of
transitions in the data record to be determined and hence measures the duration of all channel openings and closings. Simultaneously, the amplitude of channel openings is measured by including, in the least squares fit, short stretches of data before and after each transition point. These amplitudes are used to re-scale the step response fitted to each transition until a best fit is achieved.

Distributions of the channel current amplitudes, open and shut times, and burst and cluster characteristics were constructed and fit using the program EKDIST as described by Colquhoun & Sigworth (1995). A fixed resolution was imposed on the data obtained with SCAN such that channel openings and closings with a duration less than two filter rise times (160-320μs) were excluded from the fit in order to exclude false events which might mistakenly be taken to be channel openings or closings.

2.4.2 Fitting channel amplitude distributions

Current amplitude distribution histograms were fit by one or the sum of two Gaussian distributions using the method of maximum likelihood (Colquhoun & Sigworth, 1995). Standard deviations for the fit were usually not constrained to be equal. The value of the mean current amplitude was calculated from the fitted distribution parameters using the equation (for \( i \) components) \( \sum a_i \mu_i \) where \( a_i \) is the relative area of the \( i \) th component of the mean \( \mu_i \).
The membrane potential in the cell-attached configuration of the patch-clamp technique is determined by the cell’s resting membrane potential and the pipette potential. The resting membrane potential was determined in these experiments by making recordings at several pipette potentials and making a plot of current against pipette potential. Assuming that the NMDA reversal potential is close to zero millivolts the cell resting membrane potential can be determined from the intersection of the extrapolated graph and the x-axis (Figure 3.2).

Open and shut time intervals determined by SCAN were used to construct distribution histograms. These results are displayed as peaked distributions of the square root of frequency verses log (shut/open) time (Blatz & Magleby, 1986; Sigworth & Sine, 1987). Shut time distributions were fit by the sum of four or five components and open time distributions by two or three exponential components by maximum likelihood fitting to the individual observations (Colquhoun & Sigworth, 1995).

2.4.3 Bursts and clusters

Previous studies by Gibb & Colquhoun, (1991; 1992) have shown that NMDA channel openings occur in short bursts grouped together in longer clusters. Bursts and clusters are defined as groups of channel openings separated by a shut time shorter than a critical time period ($T_{\text{crit}}$). $T_{\text{crit}}$ was calculated for bursts from the 2nd shortest and 3rd shortest components of the shut time distribution and for clusters from the 3rd shortest and 4th shortest components of the shut time
distribution by iteratively solving for $T_{crit}$ in Equation 2.1 (Colquhoun & Sakmann 1985).

$$1 - \exp\left(-\frac{T_{crit}}{\tau_s}\right) = \exp\left(-\frac{T_{crit}}{\tau_f}\right) \text{(Equation 2.1)}$$

where $\tau_s$ and $\tau_f$ are (respectively) the time constants of the slower and faster exponential components obtained from the fit of the shut time distribution.

### 2.5 Voltage ramp protocol

Current against voltage relationships were constructed in some experiments by using a voltage ramp protocol generated by software provided by Prof. D. Colquhoun (CJUMP4). Four voltage ramp trials were carried out before during and after drug application and each set averaged. The net current against voltage plot was constructed by subtracting the drug average from the averages of the trials before and after drug application. Each ramp protocol consisted of a 3 second ramp from -60mV to +40mV, then to -100mV and back to -60mV.

#### 2.5.1 Fitting voltage ramps

Current against voltage relationships were fit using a modified Goldman-Hodgkin-Katz (GHK) equation (Equation 2.2) assuming a simple open channel blocking mechanism for Mg$^{2+}$ (Nowak et al., 1984; Ascher & Novak, 1988). The net membrane current as a function of the membrane potential, $I(V)$, was assumed to be
due to the inward movement of external monovalent ions of concentration \([M^+]_o\), and outward movement of internal monovalent ions of concentration \([M^+]_i\). Fitting was simplified by not explicitly including a term for divalent permeation but instead assumed that, over the potential range of the experiment, divalent ion movement would be exclusively inward producing an offset \((E_{off})\) in the reversal potential which was included as a variable in the fitting process.

\[
I(V) = \frac{0.001 \frac{F^2 V}{RT}}{1 - \exp\left(-\frac{FV}{RT}\right)} \left\{ P_m[M]_i - P_m[M]_o \exp\left(-\frac{FV}{RT}\right) \right\} \left(1 - P_m(V)\right)
\]

**Equation 2.2**

where \([M]_o\) is the concentration of external monovalent cations, \([M]_i\) is the concentration of internal monovalent cations, \(P_m\) is the permeability coefficient for the internal monovalent cations and \(P_{mo}\) is the permeability coefficient for the external monovalent cations. The constants are; \(F\), the Faraday constant \((9.65 \times 10^4 \text{ C mol}^{-1})\); \(R\), the gas constant \((8.32 \text{ J K}^{-1}\text{ mol}^{-1})\) and \(T\) the absolute temperature \((293\text{K at room temperature})\). The fraction of current blocked by \(\text{Mg}^{2+}\) was calculated as

\[
p_{\text{Mg}}(V) = \frac{[\text{Mg}]}{[\text{Mg}] + K_B(V)}
\]

**Equation 2.3**

where \(K_B(V) = K_B(0) \exp(V/H)\) with \(K_B(0)\) being the equilibrium constant for block by magnesium at zero mV membrane potential and \(H\) being the change in membrane potential (in mV) required to produce an e-fold change in the equilibrium constant. I-V relations were fitted assuming contaminant \(\text{Mg}^{2+}\) concentration in control solutions of 4µM (Gibb & Colquhoun 1992). Thus five parameters: \(E_{off}\), \(P_{mo}\), \(P_{mo}, K_B\) and \(H\) were varied by a non-linear least squares fitting routine (CJFIT, kindly provided by Prof. D. Colquhoun) to fit the modified GHK equation to the I-V
curves. The voltage dependence of the dissociation equilibrium constant can also be described by:

\[ K_a(V) = K_a(0) \exp \left( \frac{\delta Z F V}{R T} \right) \]  \hspace{1cm} \text{Equation 2.4}

where \( \delta \) is the fraction of the membrane voltage that the blocker senses at the binding site and so is taken as a measure of where the blocker binds, and \( z \) is the valence of the blocker.

The GHK equation was used to fit the NMDA current voltage relationships because this has previously been shown to provide a good description of the NMDA current-voltage relation (Forsythe & Westbrook, 1988; Burnashev et al., 1995; Schneggenburger, 1996) and so provides a reasonably unconstrained way to quantify the parameters for Mg\(^{2+}\) block of the channel given the assumptions inherent in this. In particular using this method to estimate the equilibrium constant and voltage dependence for Mg\(^{2+}\) block assumes a \( P_{\text{open}} \) of 1 for the NMDA channels. In addition Schneggenburger (1996) recently showed that when using the GHK equation to describe the fractional contribution of divalent ions to the current through NMDA-channels this method showed close agreement with results using direct methods of measurements of the Ca\(^{2+}\) current using the fluorescent dye fura-2.

2.6 Current fluctuation analysis

Current fluctuation or noise analysis is a technique for extracting information from macroscopic currents about the underlying unitary channel behaviour. Stationary noise analysis was devised by Katz & Miledi (1970) and provided the first estimates
of single channel conductance (Katz & Miledi, 1972; Anderson & Stevens, 1973). Sigworth (1980) extended noise analysis to the study of non-stationary noise. With the introduction of the “giga-ohm seal” (Hamill et al., 1981) and the associated increase in signal to noise ratio afforded by the use of modern patch-clamp techniques, its popularity as an investigative tool has declined because in many situations single channel currents can now be observed directly. However it still provides a useful method of looking at the behaviour of low conductance somatic and synaptic ion-channels (Cull-Candy & Ogden, 1985; Traynelis et al., 1993; Momiyama et al., 1996b) and non-stationary noise has been used to estimate the probability of channel opening (Silver et al., 1996).

2.6.1 Stationary noise analysis

Stationary noise analysis was used to analyse ion channel currents induced by application of 20μM NMDA and 5μM glycine. The steady-state macroscopic current can be thought of as comprising of two signals; the mean DC current and fluctuations around this mean. The DC component of the current was removed by high pass filtering at 1Hz (Barr & Stroud 8 pole Butterworth) and this filtered signal was then amplified and low-pass filtered at 800Hz (8 pole Butterworth).

The analysis of data gathered in this way is based upon the following: the whole-cell current \( I \) for a cell containing \( n \) ion channels each with a single channel current amplitude of \( i \) and an open probability \( p \) can be given by the equation,

\[
I = i \cdot n \cdot p
\]
The variance of the current fluctuations about this mean value is given by;

\[ \sigma^2 = i^2 \cdot n \cdot p(1 - p) \]

combining these two equations gives;

\[ \sigma^2 = i \cdot I - I^2 / n \]

and if \( p \ll 1 \) then;

\[ \sigma^2 = i \cdot I \]

the variance is therefore directly proportional to the mean current at low open probabilities. If the cell resting membrane potential (Vm) and the current reversal potential (Er) for the ion channel are also known, then the single channel conductance can be calculated from;

\[ \gamma = (\sigma^2 / I) / (V_m - Er) \]

Power spectra were calculated by Fourier transform of 1 second long segments of data for baseline and agonist induced currents using the program SPAN (kindly provided by J Dempster, Strathclyde Electrophysiology Software). The net power spectrum was calculated by subtracting the mean of 32-64 baseline spectra from the mean of 32-64 spectra calculated from noise segments in the presence of agonist. Net spectra were fit with the sum of two Lorentzian components and the single channel current estimated from the net agonist-induced current variance and from the fit of the power spectra.
Chapter 3 Modulation of the single channel properties of NMDA receptors by activation of metabotropic glutamate receptors

3.1 Summary

2. NMDAR single channel currents were evoked in cell-attached patches made from granule cells in the dentate gyrus of rat hippocampal slices. Steady state recordings were made at several holding potentials and the channel amplitudes and open and shut times measured.

3. mGluRs were activated by bath applying the selective agonist 1S, 3R ACPD for a minimum of ten minutes. NMDAR channel openings were again recorded at several holding potentials and analysis of channel openings carried out in order to see if the channel’s opening behaviour was modulated in any way by mGluR activation.

4. Surprisingly, mGluR activation caused a clear reduction in the slope conductance of the NMDAR-channels in combination with an attenuation in the mean open time, mean burst length and mean cluster length.

3. Experiments involving the application of inhibitors of kinases and phosphatases involved in protein phosphorylation were carried out to see if the mechanism of this modulation could be occluded. These experiments
suggested that inhibition of Ca^{2+}/calmodulin phosphatase 2B (calcineurin) occluded the mGluR-mediated modulation.

4. Perforated vesicles were formed from hippocampal granule cells. Application of 1S, 3R ACPD to these patches did not alter NMDA receptor-channel properties.

5. Channels were recorded from inside-out, crammed, and outside-out patches and a comparison made between single channel parameters in each configuration, in particular to see if patch excision caused a change in channel properties. While the results of the patch cramming experiments are equivocal, comparison of inside-out and outside-out patch data suggests that channel properties change on formation of excised patches resulting in longer open times compared to the cell-attached configuration.
3.2 Introduction

There have been a number of studies which have made detailed analysis of the openings of the NMDA receptor-channel using both outside-out and cell-attached configurations of the patch-clamp technique (Howe et al., 1991; Gibb & Colquhoun, 1991; Gibb & Colquhoun, 1992; Kleckner & Pallotta, 1995).

These studies have shown that the NMDA receptor-channel shows very complex characteristic patterns of opening. These studies have quantified the channel’s conductance and distinctive sub-conductance levels, its open and shut times, open frequency, open probability and burst and cluster lengths.

Several studies have suggested that these single channel parameters are subject to influence by phosphorylation. The length of openings along with the burst and cluster lengths can be prolonged and open probability (P_{open}) is reduced by applying inhibitors of protein phosphatases (Lieberman & Mody, 1994; Wang et al., 1994). Similarly, direct exogenous application of protein phosphatases 1 and 2A to inside-out patches results in a reduction of P_{open} (Wang et al., 1994). PKC has been shown to increase open probability and in some cases to reduce Mg^{2+} sensitivity of the channel (Chen & Huang, 1992 but also see Wagner & Leonard, 1996).

Therefore there is considerable evidence to suggest that modulation of macroscopic currents referred to in Section 1.2.7 is due to a change in the
underlying single channel openings. The studies mentioned above did not report any changes in the NMDAR single channel conductance. There have however been reports suggesting phosphorylation induced changes in single channel conductance of non-NMDA receptors (Kamboj, 1996) and 5-HT3 receptors (Van Hooft & Vijverberg, 1995) and also a report of a change in channel conductance of NMDARs due to patch excision from the cell membrane (Clark et al., 1996).

There are numerous physiological mechanisms which may activate these intracellular modulators including other membrane bound receptors (Chen & Huang, 1991; Raman et al., 1996). One likely group of candidates which may be involved in any modulation of NMDARs are the mGluRs. Activation of mGluRs may activate numerous intracellular processes (see Section 1.4) which could be responsible for a direct or indirect modulation of NMDARs. As already mentioned in Section 1.4.4, mGluRs have been shown to modulate whole-cell and synaptic NMDA-mediated responses.

In this chapter I present results from recordings of single channel currents in cell-attached patches from granule cells of the rat hippocampus. Activation of mGluRs using the specific agonist 1S, 3R ACPD caused a surprising change in the single channel properties of the receptor.

Some of this work has previously been published (Contractor & Gibb, 1995a; Contractor & Gibb, 1995b).
3.3 Results

3.3.1 Stability of channel behaviour

Experiments designed to obtain cell-attached recordings containing NMDA receptor channel openings from granule cells had a low success rate. Patches seemed to contain either no channel openings or else openings would be seen for a short while after seal formation and then would disappear completely. This was surprising since NMDA channel activity is stable for many minutes in outside-out patches (for example see Gibb & Colquhoun, 1991). The mechanism for this lack of activity is unknown and the reason that it sometimes did not occur (and channel openings could be observed in patches for long periods of time) is also difficult to ascertain. However in some later experiments the use of pipette solutions containing zero Ca\(^{2+}\) (described in Section 3.3.5) greatly increased the success rate of obtaining patches with long-lived channel activity.

3.3.2 Slope conductance of NMDA channels in cell-attached patches

Recordings were made of channel openings in cell-attached patches with agonist (5\(\mu\)M NMDA and 5\(\mu\)M glycine) included in the patch pipette along with 1mM Ca\(^{2+}\). Figure 3.1 illustrates representative stretches of data showing channel openings recorded from a cell-attached patch during control recording and after 50\(\mu\)M 1S, 3R ACPD had been applied to the slice. Channel amplitude histograms were constructed at different pipette potentials (Figure 3.2a) and mean channel
Figure 3.1 Shows representative stretches of data from the same cell attached patch before (left panel) and after (right panel) 50 μM IS, 3R ACPD had been applied to the slice for approximately 10 minutes. NMDA channel openings due to inclusion of 5μM NMDA and 5μM glycine in the patch pipette are seen as downward deflections. Both stretches are recorded at equivalent membrane potentials of -75mV. Time course fitting of the data file from which these stretches were taken was used to construct amplitude distribution histograms shown underneath. During control the main conductance level is 47.2pS and the sub-level is 34.8pS. After application of ACPD the main level is 36.5pS and the sub-level is 32.3pS. The mean channel conductance for this patch during control recording was 47 pS and 34 pS after activation of mGlurRs. If the standard deviations of the fit are constrained to be equal, during control the main level is $3.57 ± 0.41$ pA (82.6%) and the sub-level is $2.78$ pA (17.4%) giving a mean current of $3.43$ pA. If the fit of the channel amplitudes during application of ACPD is also constrained to have equal standard deviations the main level is $2.79 ± 0.5$ pA (78.3%) and the sub-level is $2.24$ pA (21.7%) which is a mean current of $2.67$ pA.
Figure 3.2a shows amplitude histograms constructed from channel openings in one patch held at four different pipette potentials. The mean of each distribution is plotted against pipette potential in Figure 3.2b to give a graph from which the cell's resting membrane potential can be found (x-axis intercept).
amplitude plotted against pipette potential (Figure 3.2b). The NMDA channel’s reversal potential is close to 0mV; this was used to estimate the resting membrane potential from the current against pipette potential plot and used to re-plot graphs of estimated membrane potential against channel amplitude. The average resting membrane potential was estimated to be -53 ± 9.5mV (mean ± SEM, n=3) during normal (control) recording in 1mM Ca$$^{2+}$$ containing Krebs.

Current against voltage curves were fitted using linear regression to measure the slope conductance of the channel. The mean slope conductance was 51 ± 4.3pS (n=3). In order to allow for differences in the cells’ resting membrane potential when combining data from different cells, data were combined after first interpolating each current-voltage curve at specific membrane potentials (Figure 3.3).

The estimated resting membrane potential after application of ACPD was -56 ± 7.2mV (n=3). The mean slope conductance of the channels showed a significant decrease from control to 40 ± 4.2pS (n=3) (Figure 3.3).

The membrane potential of the cell sometimes changed when 1S, 3R ACPD was initially applied to the slice. However, this had stabilised by the time recordings were made. To check that the membrane potential was not changing while recordings were being made, channel amplitude stability plots were constructed (Figure 3.4) from which it is clear that the membrane potential during recording periods showed no tendency to drift.
Figure 3.3 Current against membrane potential plot for NMDA channels activated by 5μM NMDA and 5μM glycine in cell-attached patch-clamp recordings from granule cells. Each point is the average single channel current at that potential from three different patches. Because of differences in cell resting membrane potentials the data from each patch has been interpolated to standard membrane potentials following linear regression of measured amplitudes in each patch. It is at once clear that the slope conductance during control recording (blue plot) is greater than that of the channels in the same patches after application of 1S,3R ACPD (red plot). The two values of slope conductance are 50.6pS during control and 39.9pS after application of 50μM ACPD.
Figure 3.4 shows an amplitude stability plot for a cell-attached recording during application of 50 μM 1S, 3R ACPD. The current amplitude changes when the pipette potential is changed however there is no drift in the channel amplitude during steady state recording indicating that the cell’s resting membrane potential is not changing during the recording period.
3.3.3 Mean open time of NMDA channels in cell-attached patches

Previous studies of NMDARs (Howe et al., 1991, Gibb & Colquhoun, 1992) have shown the open time of NMDARs to be voltage dependent due to blockade of the ion channel by residual Mg$^{2+}$. When divalent ions are buffered to very low levels using EDTA the NMDA channel open times are voltage independent (Gibb & Colquhoun, 1992).

Distributions of open time or open periods were constructed and fitted with the sum of one to three exponential components. Channel open periods were defined as the total contiguous opening of a channel. These give a better indication of the channel opening behaviour than open times as they avoid division of individual openings into several openings with closely spaced channel amplitudes caused by fluctuations due to open channel noise.

The mean of the distribution of open times was calculated and plotted against membrane potential. Again, when combining data from different cells, in order to allow for differences in the resting membrane potential between cells, open time data were interpolated to several standard membrane potentials. Figure 3.5 shows the relationship between the membrane potential and mean open time of the NMDA channels from data combined from three cell-attached patches. There is a clear reduction in open time of the NMDA channel at all potentials after activation of mGluRs.
Figure 3.5 shows mean open time (log scale) of NMDA channels from three different patches which have been interpolated to set estimated membrane potentials and averaged. The graph shows the relationship between open time and membrane potential during control recording (blue plot) from cell-attached patches and after application of 50μM ACPD (red plot).
However there is no difference in the slope of the two graphs suggesting that channel openings are shortened at all membrane potentials to the same degree, which might further suggest that although this change in open time could be due to an increase in the affinity of Mg$^{2+}$ for the channel, it is not due to an increase in the voltage sensitivity of the Mg$^{2+}$ block as this would be expected to change the voltage-dependence of the open times.

### 3.3.4 Burst and cluster length

Shut time distributions were constructed for the data at each membrane potential. $T_{\text{crit}}$ calculations were made from these using Equation 2.1 as described in Section 2.3.1. Figure 3.6 shows inset the shut time distribution from which $T_{\text{crit}}$ was calculated. Representative burst length distributions are shown for channels from the same patch before and after application of ACPD.

An average of the mean burst length of NMDA channels was calculated at an estimated membrane potential of -100mV. During control recording the mean burst length was $3.75 \pm 0.79\,\text{ms}$ (n=3). This was reduced to $1.24 \pm 0.16\,\text{ms}$ (n=3) during application of ACPD. The average $T_{\text{crit}}$ value was shorter but not significantly different between the two recording conditions (control; $1.12 \pm 0.31\,\text{ms}$, following ACPD; $0.65 \pm 0.23\,\text{ms}$, n=3) and there was no significant change in either the overall opening frequency (control; $8.56 \pm 3.6\,\text{s}^{-1}$, following ACPD; $11.5 \pm 7.33\,\text{s}^{-1}$, n=3) or in the burst $P_{\text{open}}$ (Control; $0.89 \pm 0.041$, following ACPD; $0.95 \pm 0.026$, n=3).
Figure 3.6 shows representative shut-time distributions (inset) for a cell-attached patch at a membrane potential of -100mV before (left panel) and after (right panel) application of 1S, 3R ACPD. Time constants calculated from these distributions were used to construct the burst length distributions (main picture in two panels). The time constants for the shut time distribution during control recording are $\tau_{1} = 0.0274\, \text{ms} \ (36.4\%); \tau_{2} = 0.993\, \text{ms} \ (44.0\%); \tau_{3} = 9.16\, \text{ms} \ (12.3\%); \tau_{4} = 141.7\, \text{ms} \ (3.2\%); \tau_{5} = 1455\, \text{ms} \ (4.2\%)$ (the mean of the distribution is 67.2ms). After application of ACPD the time constants of the shut time distribution are $\tau_{1} = 0.0354\, \text{ms} \ (44.4\%); \tau_{2} = 0.625\, \text{ms} \ (19.1\%); \tau_{3} = 4.23\, \text{ms} \ (19.8\%); \tau_{4} = 64.6\, \text{ms} \ (19.4\%); \tau_{5} = 303.2\, \text{ms} \ (2.6\%)$ (the mean of the distribution is 21.2ms). The time constants of the burst length during control are $\tau_{1} = 0.626\, \text{ms} \ (5.7\%); \tau_{2} = 5.59\, \text{ms} \ (94.3\%) \ (\text{mean} = 5.31\, \text{ms})$. After application of ACPD time constants for the burst length distribution are $\tau_{1} = 0.134\, \text{ms} \ (33.3\%); \tau_{2} = 1.21\, \text{ms} \ (46.5\%); \tau_{3} = 4.90\, \text{ms} \ (20.2\%) \ (\text{mean burst length} = 1.59\, \text{ms})$. 
Similarly the average of the mean cluster length was calculated at -100mV membrane potential. This gave an average value during control recording of 16.6 ± 6.21ms (n=3). This was reduced to 5.76 ± 2.78ms (n=3) during application of ACPD. Again there was no significant difference in the cluster \( P_{\text{open}} \) (Control; 0.62 ± 0.09, following ACPD; 0.59 ± 0.09, n=3).

3.3.5 Calcium influx through NMDA receptors

Cell-attached recordings were made from cells using a pipette solution containing 0mM Ca\(^{2+}\) and 10mM EGTA to see if Ca\(^{2+}\) influx through NMDA receptors was a requirement for the modulation reported in the previous section. In the one recording shown in Figure 3.7 the reduction in slope conductance seemed to be independent of Ca\(^{2+}\) influx, however the removal of Ca\(^{2+}\) seemed to prevent the reduction in mean open time previously seen when recording with a pipette solution containing 1mM Ca\(^{2+}\).

3.3.6 Cyclosporin A prevention of mGluR mediated modulation

Given the results of Lieberman & Mody, (1994) and others (discussed in Section 1.2.7) the change in receptor kinetics seen after activation of mGluRs may be caused by an intracellular mechanism leading to an alteration in the NMDAR phosphorylation state. Inhibitors of intracellular enzymes were therefore used to test this idea.
Figure 3.7 shows the current against voltage plot for NMDA activated channels from a cell-attached patch in which 0 mM Ca$^{2+}$ and 10 mM EGTA were included in the patch pipette. The reduction in slope conductance seems to be not dependant on Ca$^{2+}$ influx through NMDA channels, however the reduction in mean open period previously seen is no longer present. The solid and dotted lines in a. are linear regressions constrained to pass through the origin.
One possible candidate which may act as an intracellular messenger in the mGluR response is calcineurin (phosphatase 2B). Therefore cyclosporin A (CsA) was used to specifically inhibit calcineurin.

Cell-attached recordings were made and after a period of control recording, 20-100nM CsA was applied to the slice for a minimum of 10 to 15 minutes. ACPD was then applied to the slice and recordings again made at several potentials. Channels in patches from slices which had been treated with CsA prior to ACPD application did not show a large decrease in their slope conductance on activation of mGluRs (Figure 3.8) as observed previously (control; cell #1 53.9pS, cell #2 51.8pS, following ACPD; cell #1 66.1pS, cell #2 45.9pS) although the resting membrane potential of the cells showed a change, depolarising from -56.5 ± 2.5mV to -39 ± 5mV (n=2).

Similarly the mean open time of the NMDA channels showed no significant change at all membrane potentials when mGluRs were activated post CsA (Figure 3.9).

The mean burst length also showed no significant change from control on ACPD application after the slices had been pre-treated with CsA (control; 4.45 ± 0.05ms, following ACPD; 4.70 ± 0.46ms, n=2; Figure 3.10). Mean cluster length was reduced to a much lesser extent in CsA treated slices after ACPD application (control; 23.9 ± 6.2ms n=2; following ACPD; 16.6 ± 5.5ms, n=2; at -70mV membrane potential) (Figure 3.10).
Figure 3.8 graph of membrane potential against channel current for two patches (data interpolated to standard membrane potentials). The slope conductance after application of ACPD to the cyclosporin A pre-treated slice (red plot) is only slightly and not significantly reduced from control recording (blue plot) unlike in previous experiments when CsA was not used to inhibit calcineurin. The solid and dotted lines are linear regressions constrained to pass through the origin.
Figure 3.9 shows plot of log of the mean open time (interpolated to standard membrane potentials) against membrane potential. The two plots represent control conditions (blue plot) and after mGluR activation in cyclosporin A pre-treated slices (red plot). Unlike experiments in the absence of CsA, the mean open time does not seem to show a significant difference at all membrane potentials. The solid and dotted lines are first order linear regressions.
Figure 3.10 Bar charts of the mean burst length and mean cluster length during control (n=3), application of 1S, 3R ACPD (n=3) and application of 1S, 3R ACPD after CsA application (n=2). The mean burst length during control was $3.76 \pm 0.790\text{ms}$ (n=3), after ACPD $1.29 \pm 0.272\text{ms}$ (n=3), after CsA and ACPD $4.70\text{ms}$ (average of n=2). The mean cluster lengths were; during control $16.6 \pm 6.22\text{ms}$ (n=3), after ACPD $3.13 \pm 1.51\text{ms}$ (n=3), after CsA and ACPD $16.6\text{ms}$. All burst and cluster lengths are calculated at a membrane potential of -70mV.
3.3.7 Inhibition of protein kinase C by calphostin C

PKC may potentially mediate inhibition of the NMDA response following mGluR activation (Kelso et al., 1992). Calphostin C, a potent and specific inhibitor of PKC (Kobayashi et al., 1989), was used to test whether mGluR’s actions involved PKC as a second messenger. Again the cell-attached mode of the patch-clamp technique was used to record NMDA activated single channel events before and after mGluR activation but in this case 200nM calphostin C was applied to the slice for approximately 20 minutes during the control period prior to ACPD application.

Figure 3.11 shows the mean current against voltage plot for channels in the same patch during control (calphostin C) and after mGluR activation. There is no change in the slope conductance of the channels after mGluR activation (control; 39.1pS, ACPD; 39.5pS).

There is however, as before, a reduction in mean open time at all the potentials tested (Figure 3.12) after application of ACPD even post calphostin C.

3.3.8 Single channel properties of NMDA receptors in inside-out and crammed patch configurations

Intracellular or cytoskeletal components may influence channels (Rosenmund & Westbrook, 1993) when recorded in the cell-attached patch-clamp configuration.
Figure 3.11 shows mean current against voltage plots for NMDA channels in the same patch during control recording (200nM calphostin C) and after application of the specific mGluR agonist 1S, 3R ACPD. Pre-treatment of the slice with this PKC inhibitor occludes the reduction in slope conductance previously seen after mGluR activation. The solid and dotted lines are linear regressions constrained to pass through the origin.

Estimated membrane potential (mV)

-90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30

Single channel current (-pA)

-3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0

37.5pS 40.0pS

- 200nM Calphostin C
- 200nM Calphostin C & 50uM ACPD
Figure 3.12 shows a plot of mean open time against membrane potential for one patch in which NMDA channels were recorded during control (calphostin C included in the bath perfusate) (blue plot) and after mGluR activation with 50μM ACPD (red plot). The mean open time is reduced at all potentials after 1S, 3R ACPD even after pre-treatment with calphostin C. Solid and dotted lines are linear regressions.
and this effect may be lost when forming the inside-out or outside-out configurations. In order to investigate this, experiments were performed to compare the properties of channels in inside-out patches when, presumably, endogenous modulators of channels are lost, with those in the same patches crammed back into the parent cell (Kramer, 1990) where intracellular modulators might once again influence channel behaviour. The main difficulty with applying this approach to mammalian neurones in brain slices is that the patch electrode is likely to kill the cell.

**Figure 3.13** shows representative current traces and amplitude histograms from the same patch in the inside-out and crammed modes at a membrane potential of -60mV. A direct comparison of channel properties in each configuration at equivalent membrane potentials shows no increase in the mean unitary chord conductance in crammed patches (55pS) compared to inside-out patches (52pS). **Figure 3.14** shows the open period histograms for the same patch again in the two different configurations at the same membrane potential. There is a slight decrease in mean open period in the crammed (4.42ms) compared to the inside-out configuration (5.45ms).

Shut time distributions (not shown) gave a $T_{\text{crit}}$ value for bursts of 3.96ms (inside-out) and 4.67 ms (crammed) from which mean burst lengths were calculated as 6.05ms (inside-out) and 5.31ms (crammed).
Figure 3.13a shows current traces from an inside out patch clamped at -60mV. Channel openings are downward in the data. The current amplitude histogram is constructed and fit using the programs SCAN and EKDIST (Colquhoun & Sigworth, 1995). Figure 3.13b shows channel openings in the same patch after it has been crammed back into the parent cell from which the patch was originally made. There is a only a slight increase in the single channel current at this membrane potential suggesting that cramming depolarised the cell.
Figure 3.14 shows open period distribution plots for channel openings from an inside out patch before; a., and after; b., the patch has been crammed back into the parent cell. The distributions are fit by two exponential components in a., and a single component in b. Mean open period for the channels in the crammed patch configuration (4.42ms) is slightly shorter than for the channels in the inside out configuration (5.45ms).
3.3.9 Outside-out patches

Outside-out patches were obtained from granule cells as described above. 5µM NMDA & 5µM glycine were applied to the patch to evoke channel openings. Unlike the inside-out or cell-attached configuration this concentration of agonist was enough to evoke considerable channel activity. The channel behaviour in this configuration is also noticeably different from the behaviour of channels in cell-attached patches.

As previously reported for NMDA channels in outside-out patches from CA1 cells (Gibb & Colquhoun, 1991) or cells in the dentate gyrus (Gibb & Edwards, unpublished) channels in these experiments also had a very obvious sub-conductance level at 40pS and a main conductance level at 50pS. Figure 3.15 shows the amplitude histogram along with inset representative stretches of data from the same outside-out patch held at -60mV.

There were also several obvious differences in channel behaviour observed in different patch-clamp configurations. The mean open time of channels in this patch at -60mV was 3.25ms (mean open period was 4.21ms), mean burst length was 8.75ms and mean cluster length was 18.9ms (Figure 3.16). Thus open times, burst lengths and cluster lengths were all longer than in cell-attached recording in agreement with previous observations from hippocampal CA1 cells (Gibb & Colquhoun, 1991; 1992) or from hippocampal granule cells (Gibb & Edwards, 1991). In these previous studies the agonist used was glutamate, but, at least in
Figure 3.15 Amplitude histogram of channel openings activated by 5μM NMDA and 5μM glycine from an outside out patch made from a dentate gyrus granule cell and recorded at a membrane potential of -60mV. The histogram and inset representative stretch of raw data show a clear sub-conductance level at about 40pS along with the main conductance level at around 50pS.
Figure 3.16 shows dwell time distributions for NMDA channels recorded in the outside out configuration. The patch was made from a dentate gyrus granule cell and held at -60mV. Channel openings were evoked by bath applying 5μM NMDA and 5μM glycine. Figure a shows the plot of open period distribution; the mean open period is 4.21ms. The mean burst length (Figure b) is 8.75ms and the mean cluster length is 18.9ms (Figure c).
cerebellar granule cells, glutamate and NMDA produce very similar channel open
times and amplitudes (Howe et al., 1991).

3.3.10 Perforated vesicles

High channel activity in perforated vesicles probably reflects the increased surface
area of membrane which is excised with these patches (Hörber et al., 1995).
Therefore low concentrations of NMDA were used (500nM) in order to minimise
double openings. Channel openings in patches were recorded in vesicles clamped
at specified membrane potentials.

Figure 3.17a shows the amplitude histogram for NMDA channels in a perforated
vesicle at -60mV. Assuming a reversal potential of 0mV the chord conductance is
57pS which is slightly higher than the mean of the slope conductances during
control recording in cell-attached patches and also higher than the chord
conductance in the outside-out configuration (Section 3.3.9). There was no change
in conductance after application of ACPD to the patch (56pS; Figure 3.17b).

Open period distributions gave a mean open period of 3.42ms during control
recording and 3.36ms after ACPD application. Again shut time distributions were
constructed and from these the burst lengths were calculated. The mean burst
length during control recording was 5.06ms and showed no change after ACPD
had been applied to the patch (5.08ms).
Figure 3.17a shows the amplitude distribution for NMDA channels recorded in a perforated vesicle at -60mV. The mean current calculated from this distribution was 3.44pA. Figure 3.17b shows the amplitude distribution plot from the same perforated vesicle after ACPD has been applied to the patch for a minimum of ten minutes. There is no change in the mean single channel current (3.37pA).
3.4 Discussion

3.4.1 Metabotropic receptor modulation of NMDA receptors

The cross-talk between different receptors in the CNS is an interesting but difficult subject to study due to the multitude of systems which may act in concert. However there have been a number of interesting studies showing interactions between different receptors; β-adrenergic receptors and NMDARs (Raman et al., 1996), μ opioid receptors and NMDARs, (Chen & Huang, 1991) mGluRs and AMPA/GABA_A (Glaum & Miller, 1994). There have also been numerous reports that mGluRs may modulate NMDA responses (Aniksztejn et al., 1992; Kelso et al., 1992; Wagner & Leonard, 1996) and NMDA mediated synaptic currents (O’Connor et al., 1994) and that they play a role in plastic changes to both non-NMDAR (Bortolotto & Collingridge, 1995) and NMDAR mediated synaptic currents (McGuinness et al., 1991; Yi et al., 1995). There is still however a considerable amount of contention as to the role of mGluRs as modulators of ionotropic glutamate receptors and the physiological relevance and molecular mechanisms involved.

Previous whole-cell current data from receptors expressed in Xenopus oocytes reported enhancement of NMDA-mediated responses via activation of PKC by mGluRs (Kelso et al., 1992). Contrary to what might be expected in the light of this work, I found in this study that the NMDA single channel current is modulated in such a manner as to expect an attenuation of the macroscopic...
current. This disparity could be due to differences in the two preparations used; the intracellular messengers and G-proteins which might couple to the mGluRs in oocytes may be different from those in cells from rat brain slice and this might be the influential aspect determining which pathways are activated by the application of an mGluR agonist.

The most curious result obtained in this study is the reduction in slope conductance of the NMDA receptor channels after mGluR activation. A change in conductance such as this has never previously been reported for the NMDAR although there is evidence that the conductance of non-NMDA (Kamboj, 1996) receptors and 5-HT$_3$ receptors (Van Hooft & Vijverberg, 1995) is enhanced by phosphorylation. It is a difficult phenomenon to explain but one could speculate that it is due to a phosphorylation induced conformational change in the receptor.

The fact that the change in conductance is a 20% reduction in slope conductance from approximately 50pS to 40pS also raises the question as to whether the modulation might be a switch in the receptor from the main conductance level, which has been well characterised to be 50pS, to a conformational state of the receptor in which the sub-conductance level (40pS) was the dominant conducting state of the receptor-channel. This is a plausible explanation although it is not obvious from the data that this is the case. As stated earlier, sub-conductance channel openings are not so obvious in this configuration as they are in outside-out patches and so it remains unclear if the modulated channel is opening to a sub-conductance of the un-modulated channel. The resolution of these recordings,
which is lower than that achievable using the outside-out configuration, could be contributory to the absence of detectable subconductance levels as Gibb & Colquhoun (1992) reported that, at least in hippocampal CA1 neurones, the mean lifetime (0.52 ± 0.1ms) is short and the proportion (18%) of openings to the sublevel conductance is relatively small in inside-out recordings.

Data from experiments using Ca²⁺ free pipette solutions seems to indicate further complications in the mechanism as the change in slope conductance seems to be independent of Ca²⁺ entry through NMDARs however the change in mean open time seems to be inhibited when calcium free pipette solution is used. This was only a preliminary result and further experiments are required to test this.

Mechanisms for modulation of NMDAR open times have previously been suggested to be a dephosphorylation of the receptor protein (Lieberman & Mody, 1994) which could enable direct modulation of the receptor by calmodulin (Ehlers et al., 1996).

There is good evidence to show that this modulation can occur by application of kinases and phosphatases to the intracellular surface of NMDARs (Wang et al., 1994; Wang & Salter, 1994; Wang et al., 1996). However it has not been established how this modulatory pathway might be controlled physiologically. The Group I mGluRs in particular are good candidates as modulators of NMDARs. These receptors are linked to many of the intracellular effector systems that modulate NMDARs (Pin & Duvoisin, 1995). Secondly, they have been shown
to be localised at the synapse, distal from the active zones (Baude et al., 1993),
and therefore may be activated to reduce the NMDA-mediated component during
times of high synaptic activity when there is release of sufficient glutamate to
“spill-over” on to these peri-synaptic sites (see Figure 6.1).

3.4.2 Inhibition of calcineurin by cyclosporin A

Cyclosporin A (CsA) combines with intracellular cyclophilins to form a complex
that specifically inhibits calcineurin (Liu et al., 1991; Fruman et al., 1994). Cyclophilins are ubiquitously expressed throughout the CNS showing particularly
high expression in the hippocampus (Goto et al., 1986; Lad et al., 1991; Bladen &
Vincent, 1994). Previous reports have shown that inhibition of phosphatases
causes an increase in mean open time, burst length and cluster length (Lieberman
& Mody, 1994). The decrease in open time, burst length and cluster length after
mGluR activation reported in this study could therefore have been a result of
increased phosphatase activity. This is a possible mechanism of action of the
group I mGluRs which release Ca^{2+} from intracellular stores via the activation of
PLC and the production of IP_{3}. The use of CsA in this study proved to be very
successful in blocking the effect of ACPD. The attenuation in single channel
properties was occluded by application of CsA prior to ACPD application
suggesting that phosphatase 2B is involved in the mGluR response.

Previous studies of NMDARs have shown that NMDAR channel openings are
voltage dependant due to a voltage dependant Mg^{2+} block (Nowak et al., 1984;
Mayer et al., 1984; Ascher & Nowak, 1988). Although the pipette solutions used in this study were made from high purity Aristar reagents to minimise divalent cation contamination, the perfusate is not completely contaminant free and there is probably a residual amount of Mg$^{2+}$ present even in the cell-attached configuration.

The reduction in mean open time seen in this study after mGluR activation could have been explained by an increase in Mg$^{2+}$ block as the NMDAR is very sensitive to open-channel blockade. Previous reports have suggested that PKC phosphorylation of NMDARs reduced Mg$^{2+}$ sensitivity of the NMDA channel (Chen & Huang, 1992). If this were the case, one could hypothesise that dephosphorylation of the receptor by phosphatases might be the cell’s mechanism of increasing the receptor-channels’ Mg$^{2+}$ sensitivity.

Although inhibition of phosphatase 2B did stop the reduction in mean open time caused by mGluRs it is not possible to conclude from these experiments whether this was due to any difference in Mg$^{2+}$ sensitivity before and after ACPD application although the lack of any change in voltage-dependence of the channel open time argues against this. Also in the light of data presented in Chapter 4, changes in Mg$^{2+}$ sensitivity seem to be an unlikely explanation. Lieberman & Mody, (1994) have previously shown that the NMDAR-channel open time can be increased by inhibiting phosphatases. However in the present study there was no significant change in the open time of the receptor when the inhibitor was applied alone. This may be due to differences in levels of basal phosphorylation of the
receptors in the two studies. Lieberman & Mody (1994) found in their study that the mean open time of channel openings in cell-attached patches from acutely dissociated granule cells was 1.27 ± 0.23ms at -60mV membrane potential. The mean channel open time in this study at the same membrane potential was 2.79 ± 0.37ms. If incremental phosphorylation of the receptor increases the open time and *vice versa* dephosphorylation reduces the open time as suggested by these two studies, then these results might suggest that the basal level of receptor phosphorylation suggested by Lieberman & Mody during their control recordings was less profound than that seen in control recordings in this study (see Figure 3.18).

### 3.4.3 Inhibition of PKC by calphostin C

Calphostin C is a specific inhibitor of PKC. Since PKC is another possible enzyme activated by group I mGluRs which could affect NMDA mediated responses (see Section 1.2.6), this inhibitor was also used to try to dissect out the mechanism of the mGluR mediated modulation. The slope conductance of the NMDAR did not show any change when mGluRs were activated after calphostin C pre-treatment. However the slope conductance measured after application of calphostin C alone (40pS) seems to have already undergone a reduction (there was no control recording in this experiment although comparison can be made to previous control data; ~50pS). This could be indicative of the receptor
Figure 3.18 illustrates the increase in mean open time of the NMDA receptor at -60mV membrane potential shown by Lieberman & Mody, (1994) using inhibitors of phosphatases and the decrease in mean open time of the receptor after mGluR activation shown in this study. This data along with data from experiments using cyclosporin A suggests that mGluRs modulate the NMDA receptor via a mechanism involving a de-phosphorylation reaction catalysed by phosphatase 2B.
phosphorylation/dephosphorylation mechanisms in the cell being in balance. Thus inhibition of PKC could result in an increase in the influence of the dephosphorylation mechanisms causing, in this case, a reduction in slope conductance. However, even though calphostin C alone seems to reduce channel slope conductance the mean open time of the NMDA channel, similar to previous experiments, was reduced by ACPD even after application of calphostin C. Thus there may be more than one mechanism inducing this modulation involving more than one enzyme which may be active physiologically or which may be recruited when others are inhibited by application of drugs.

There have been two previous studies looking at modulation of NMDA activated single channel openings by the action of agonists acting on other membrane receptors. Kovalchuk et al., (1993) showed that application of high concentrations of glutamate caused an increase in open probability and an increase in open time of the NMDARs in cell-attached patches. Although the authors ascribe this to mGluR induced reduction of the voltage-dependent Mg$^{2+}$ blockade of the receptor, there is no evidence to suggest that this is an mGluR mediated effect (since they use a non-selective agonist) and there is also no data to support the idea of a change in Mg$^{2+}$ sensitivity.

Another study reported by Yu et al., (1995) produced results which were different to those seen in this study. The data reported no modulation of NMDA single channels in cell-attached patches but surprisingly saw a decrease in NMDA open
probability in outside-out patches which they attributed to a local membrane delimited modulatory action.

In results reported in Section 3.3.10 of this thesis, application of ACPD was made to excised perforated vesicles. These patches are presumed to have a greater amount of intracellular contents and patch membrane associated with them than outside-out patches and therefore possibly some of the modulatory mechanisms of the cell may be present in this configuration. Channels in patches recorded in this configuration might be expected to display the low $P_{\text{open}}$ (compared to the outside-out configuration) and the lack of double openings seen in cell-attached patches. However this was not the case and in addition, application of mGluR agonist produced no change in the chord conductance of the receptors or in the open time, burst or cluster lengths.

3.4.4 Other patch-clamp configurations

Recordings were also made in outside-out, inside-out and crammed patch configurations. These recordings were made to test the idea that channel properties might be influenced by the recording configuration and excision of a patch might lead to the loss of modulatory mechanisms which influenced these parameters.

The table below summarises the data from patches in the different configurations tested. Although it is difficult to make conclusions from such a small sample there seems to be some differences between the properties of the receptors observed in
each configuration. The most interesting comparison is that of the open time and open period of the NMDAR-channel which, within the constraints of this small sample, seem to be greater in excised patches. A previous study by Covarrubias & Steinbach (1990) reported a decrease in mean open time and burst duration of the muscle nAChR after patch excision. However Clark et al., 1997 failed to resolve any significant change in mean open time of NMDAR-channels from cerebellar granule cells reporting a mean open time of 2.4 ± 0.3 (n=4) for channels in the cell-attached configuration and 3.0 ± 0.3 (n=7) for channels in outside-out patches (both measured at -60mV).

<table>
<thead>
<tr>
<th></th>
<th>Cell-attached</th>
<th>Inside-out</th>
<th>Crammed</th>
<th>Outside-out</th>
<th>Perforated</th>
</tr>
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<tbody>
<tr>
<td>Conductance (pS)</td>
<td>50.8±4.33*</td>
<td>52.7</td>
<td>55.0</td>
<td>51.3</td>
<td>57.3</td>
</tr>
<tr>
<td>Open time (ms)</td>
<td>2.79±0.373*</td>
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<td>3.55</td>
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<tr>
<td>Open period (ms)</td>
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<td>5.45</td>
<td>4.42</td>
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<td>3.42</td>
</tr>
<tr>
<td>Burst length (ms)</td>
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<td>6.16</td>
<td>5.31</td>
<td>8.75</td>
<td>5.06</td>
</tr>
<tr>
<td>Cluster length (ms)</td>
<td>19.8±6.38*</td>
<td>47.2</td>
<td>7.39</td>
<td>18.9</td>
<td>11.75</td>
</tr>
</tbody>
</table>

* denotes n=3 for the data from cell-attached patches.

Table 3 compares single channel properties of NMDARs from patches in five different patch-clamp configurations. There seems to be some small differences in the properties of the channels tested in each different configuration. All data are from patches at a membrane potential of -60mV.
3.5 This study in the context of previous work

Previous studies have reported mGluR involvement in potentiation (Aniksztejn et al., 1992; Kelso et al., 1992; Wang & Daw, 1996) and attenuation (Colwell & Levine, 1994) of NMDAR mediated responses and mGluR potentiation (O' Connor et al., 1994) or depression (Yi et al., 1995) of NMDA mediated synaptic currents. NMDAR modulation has also been suggested to occur by dephosphorylation of the receptor (Lieberman & Mody, 1994; Wang et al., 1994). This study points to a possible physiological mechanism by which modulation of NMDAR mediated responses by endogenous mechanisms might occur at the synapse.

Modulation of the NMDARs at the synapse may be an important mechanism by which postsynaptic depolarisation, calcium influx and synaptic integration is controlled during times of high synaptic activity. This modulatory mechanism would also influence synaptic strengthening thought to be important in learning and memory formation and could also have a role in anoxic injury after acute trauma or stroke. If mGluR modulation of NMDARs is a ubiquitous phenomenon throughout the CNS, mGluRs may play a crucial role in future as targets for drug development.
3.6 Future directions

This project has produced a number of intriguing and interesting ideas which need further study. The most interesting phenomena is the change in slope conductance of the NMDARs. From these studies it is suspected that this is caused by a group I mGluR mediated dephosphorylation of the NMDA receptor protein.

To further isolate this mechanism, molecular genetic techniques are needed to look at recombinant NMDARs co-expressed in cell lines with either mGluR1 or mGluR5 to establish which subunits of the NMDAR might be involved. More sophisticated experiments would involve generating chimeras of these subunits and site-directed mutagenesis to look for the sites of modulation on the protein.

This study suggests that the phosphatase involved in this modulation is calcineurin and that this alone might cause the changes in NMDAR single channel properties. However the anomalous result obtained with the PKC inhibitor calphostin C, which occluded the mGluR mediated change in slope conductance but did not affect changes in open time and burst length, suggests a more complicated mechanism in which different kinases and phosphatases might be involved.

Biochemical studies using phosphatase labelling are also needed to establish directly that this modulation does involve dephosphorylation of the receptor protein.
It would also be interesting to see whether this phenomenon manifested itself at the synapse. It would be expected from this study that the NMDAR mediated component of the synaptic current would be reduced in amplitude and time course when postsynaptic mGluRs were activated.

Finally in the light of studies by Ehlers et al., (1996), in which they showed that direct binding of calmodulin to the alternatively spliced C-terminal exon causes a change in receptor kinetics, experiments to look at the involvement of calmodulin in this modulation could be performed.
Chapter 4 Magnesium sensitivity and divalent permeability of NMDA receptors following metabotropic glutamate receptor activation

4.1 Summary

1. Perforated patch whole-cell recordings were made from granule cells in the dentate gyrus of rat hippocampal slices. NMDA and glycine were bath applied to induce inward currents.

2. Slow voltage ramps were performed on the cells before, during and after agonist application and current against voltage plots constructed by first subtracting the average of the current-voltage relationships before and after NMDAR agonist application from the average of the current-voltage relationship recorded during agonist application. Agonist applications were repeated in the presence of 50μM and 100μM magnesium (Mg²⁺) in the extracellular solution. Current against voltage plots were again constructed.

3. ACPD was applied to the slice for a minimum of ten minutes to activate mGluRs. NMDAR agonist applications were again made in the presence of different [Mg²⁺]_{ext} in the continued presence of ACPD and current against voltage plots constructed as before using the voltage ramp protocol.
4. The I/V relationships were fit using a modified Goldman-Hodgkin-Katz (GHK) equation and Mg$^{2+}$ sensitivity examined by comparing the $K_B$ and $\delta$ values for the Mg$^{2+}$ block of the NMDAR-channels before and after mGluR activation.

5. No differences were seen in these values and it is concluded that mGluRs reduce NMDAR-channel open time by a mechanism other than an increase in the Mg$^{2+}$ sensitivity of the receptor-channel.

6. In a separate series of experiments the current-voltage relationship was examined in the presence of different milli-molar concentrations of barium (Ba$^{2+}$) in the extracellular solution. The GHK equation can be used to describe the relationship between the current reversal potential ($E_{rev}$) and the $[\text{Ba}^{2+}]_{\text{Ext}}$ to give a permeability ratio of divalents to monovalents ions ($P_{\text{Ba}^{2+}}/P_{\text{M}^+}$) before and after activation of mGluRs.

7. Following mGluR activation $P_{\text{Ba}^{2+}}/P_{\text{M}^+}$ was significantly reduced. However the values obtained for $P_{\text{Ba}^{2+}}/P_{\text{M}^+}$ were surprisingly high giving some doubt as to whether mGluR activation reduced the divalent permeability of the channel. However the experiments are suggestive that this is the case and further experiments are required to test this idea.
4.2 Introduction

4.2.1 Mg$^{2+}$ sensitivity of the receptor

The neurotoxic potential of the NMDAR-channel conferred upon it by its high Ca$^{2+}$ permeability is controlled by a number of processes. The receptor-channel is unique amongst ion channels in its ability to be blocked by micro-molar concentrations of external Mg$^{2+}$ ions (first described by Ault et al., 1980). Mg$^{2+}$ ions cause an open channel block by entering the channel pore and blocking the flow of ions through the channel. The binding site for Mg$^{2+}$ in the channel has been found using site directed mutagenesis studies (Bumashev et al., 1992) which showed that an asparagine to glutamine substitution in the M2 region of the NR2A subunit of the receptor (N595Q) strongly reduced Mg$^{2+}$ block and increased Mg$^{2+}$ permeability. This is the equivalent site that in the NR1 subunit isoform of the receptor controls Ca$^{2+}$ permeability.

A simple scheme for open channel block was first proposed by Armstrong (1969) to describe tetraethylammonium (TEA) block of squid axon K$^+$ channels and subsequently the mechanism of the block was developed by Adams (1975; 1976) and others. Neher & Steinbach (1978) provided the first single channel records showing open channel flickery block due to the blocking effect of the local anaesthetic QX-222 on the AChR-channel. The model for this blocking mechanism suggests a stochastic process as single molecules of the drug enter and leave the channel pore blocking the channel but preventing it from closing; the
agonist remains bound to the receptor until eventually, following unblocking, the agonist dissociates. This scheme can be written as:

\[
\begin{align*}
R_{\text{closed}} & \xrightleftharpoons[\alpha]{\beta} R_{\text{open}} \\
& \xrightarrow[k_{\text{on}}[\text{Mg}^{2+}]]{k_{\text{off}}} R_{\text{blocked}}
\end{align*}
\]

From this scheme the dissociation equilibrium constant for Mg\(^{2+}\) can be found and \(\delta\), the fraction of the membrane voltage field the blocker senses at the binding site, determined.

This is a good model for the channel block of acetylcholine receptors by local anaesthetics up to concentrations of around 40\(\mu\)M (Neher, 1983) beyond which, the predictions of the model breakdown. However it is only an approximation for the block of NMDARs by Mg\(^{2+}\) which differs from the predictions of the model in a number of ways.

Firstly the model does not take into account the multiple closed and open states of the NMDAR-channel (Gibb & Colquhoun, 1992). Secondly the model predicts an increase in burst duration so that the total charge transfer during a receptor activation in the presence of Mg\(^{2+}\) is the same as that for a receptor activation in the absence of Mg\(^{2+}\). This observation is true for the local anaesthetic block of the ACh channel at low local anaesthetic concentrations, but not true for the Mg\(^{2+}\) block of the NMDA channel. Mg\(^ {2+}\) block actually reduces the burst duration and therefore reduces the charge transfer (Nowak et al., 1984; Ascher & Nowak, 1988). This reduction in burst length has been explained by the channel being able
to undergo the normal closing transition while $\text{Mg}^{2+}$ is bound leaving the $\text{Mg}^{2+}$ ion trapped in the channel (Benveniste & Mayer, 1995).

The voltage dependant $\text{Mg}^{2+}$ blockade of the channel is not entirely uniform throughout the CNS. Monyer et al. (1992) showed that $\text{Mg}^{2+}$ sensitivity was dependant on the NR2 isoform of the receptor. Block of recombinant receptors consisting of NR1 and NR2C subunits was considerably weaker than the block of other subunits suggesting that native receptors may show functional diversity in different mature brain areas as well as during changes in subunit expression during development (Farrant et al., 1994; Monyer et al., 1994; Momiyama et al., 1996a).

$\text{Mg}^{2+}$ sensitivity of NMDARs can also be reduced by the action of PKC in some cells (Chen & Huang, 1992). If phosphorylation is a mechanism for decreasing $\text{Mg}^{2+}$ sensitivity it is feasible that dephosphorylation by the action of phosphatases might increase the $\text{Mg}^{2+}$ sensitivity. Although a number of studies have shown that the mean open time of NMDARs is reduced either by direct or indirect phosphatase activity (Lieberman & Mody, 1994; Wang et al., 1994; Contractor & Gibb, 1995a) it has not been established whether this is due to a change in block by $\text{Mg}^{2+}$.

In order to test the hypothesis that the reduction in mean open time of NMDAR single channel events shown in Chapter 3 of this thesis was due to a change in $\text{Mg}^{2+}$ sensitivity, experiments measuring $\text{Mg}^{2+}$ block were performed on these cells.
4.2.2 Divalent permeability of the NMDA receptor

The actions of calcium as an intracellular messenger and the relatively high permeability of the NMDAR-channel to divalent cations (Ascher & Nowak, 1988; Forsythe & Westbrook, 1988; Schneggenburger, 1996) enable activation of the receptor to initiate many of the processes thought to be involved in synaptic plasticity (Bliss & Collingridge, 1993), excitotoxic neuronal cell death and neurodegenerative disease (Meldrum & Garthwaite, 1990) & epilepsy (Clark et al., 1994).

The first studies to show Ca^{2+} influx through NMDARs used Ca^{2+} sensitive dyes (McDermott et al., 1986; Mayer et al., 1987) but the first quantitative study of divalent permeability of the NMDA receptor was made by Mayer & Westbrook (1987) who measured shifts in reversal potential (E_{rev}) and fit the data to the GHK voltage equation.

A similar approach was taken in this study. Shifts in E_{rev} were estimated from the current against voltage plots in the presence of 0.5mM and 5.0mM Ba^{2+} and the permeability ratio P_{Ba^{2+}}/P_{M^{+}} calculated from the GHK equation as follows:

\[
E_{\text{rev}} = \frac{RT}{F} \ln \frac{-b + \sqrt{b^2 - 4ac}}{2a} \quad \text{Equation 4.1}
\]

where
\[ a = K_i + 4 \frac{P_{Ba}}{P_K} Ba_i^{2+} + \frac{P_K}{P_{Na}} Na_i^* \]

We assume that \( Ba_i^{2+} = \text{zero} \) and \( P_K = P_{Na} = P_M \) then \( a = M_i = K_i + Na_i \)

also:

\[ b = K_i - K_o + \frac{P_{Na}}{P_K} (Na_i - Na_o) \]

If we assume \( K_i = Na_o \) and \( K_o = Na_i \) and \( P_K = P_{Na} = P_M \) then \( b = \text{zero} \)

also:

\[ c = -K_o - \frac{P_{Na}}{P_K} Na_o^* - 4 \frac{P_{Ba}}{P_K} Ba_o^{2+} \]

again assuming \( P_K = P_{Na} = P_M \)

\[ c = -K_o^* - Na_o^* - 4 \frac{P_{Ba}}{P_M} Ba_o^{2+} = -M_o + 4 \frac{P_{Ba}}{P_M} Ba_o^{2+} \]

For two reversal potentials \( E_1 \) and \( E_2 \)

If we substitute in Equation 4.1: \( A = \frac{RT}{F} \) and \( B = \frac{\sqrt{-4ac}}{2a} \)

\[ E_{\text{Rev}} = A \ln(B) \]

\[ E_1 - E_2 = A \ln(B_1) - A \ln(B_2) \]

Substituting back into Equation 4.1 and rearranging

\[
\exp \left( \frac{2E_1 - 2E_2}{A} \right) = \frac{c_1}{c_2} = \frac{M_o + 4 \frac{P_{Ba}}{P_M} Ba_1^{2+}}{M_o + 4 \frac{P_{Ba}}{P_M} Ba_2^{2+}}
\]

Rearranging
\[
\frac{P_{Ba}}{P_M} = \frac{M_o \left\{1 - \exp\left[ \frac{2(E_1 - E_2)}{RT/F} \right]\right\}}{\exp\left[ \frac{2(E_1 - E_2)}{RT/F} \right] \cdot 4Ba^{2+} - 4Ba^{2+}} \tag{Equation 4.2}
\]

From \textbf{Equation 4.2} the relative divalent permeability can be calculated by substituting the measured value for the shift in reversal potential on changing the barium concentration from 0.5mM - 5mM.
4.3 Results

4.3.1 Control responses to NMDA & glycine in the presence of different Mg$^{2+}$ concentrations.

Bath applied 20μM NMDA and 5μM glycine evoked an average inward current in dentate gyrus granule cells clamped at -60mV (1mM Ba$^{2+}$ in the external) of 17.3 ± 3.23pA (n=9) (Figure 4.1a). Even in nominally Mg$^{2+}$ free external solutions in most instances the current against voltage relationship, which was generated by a voltage ramp protocol (see Section 2.5), showed a characteristic outward rectification suggestive of Mg$^{2+}$ block at hyperpolarised potentials (Nowak et al., 1984) (see Figure 4.1b)

Two further agonist applications in the presence of progressively greater concentrations of Mg$^{2+}$ (50μM and 100μM) produced responses which, as expected, were attenuated and whose current voltage relationship displayed a greater degree of voltage dependency (see Figure 4.2). The current voltage relationships were fit as described in Section 2.4.1 using a modified Goldman-Hodgkin-Katz (GHK) equation and taking the simple open channel blocking model to be true as a first approximation in this case (Neher & Steinbach, 1978). Figure 4.2 shows the fit to each of the I-V relationships obtained from voltage ramps performed in nominally 0μM, 50μM and 100μM Mg$^{2+}$ for one cell. Previous studies have shown the contaminant [Mg$^{2+}$] in the solution is in
Figure 4.1a shows a perforated patch whole cell response in a hippocampal dentate gyrus granule cell from a P14 Sprague-Dawley rat. Current is activated by bath application of 20µM NMDA and 5µM Glycine. The external solution contains 1mM Ba\(^{2+}\) and is nominally Mg\(^{2+}\) free. The deflections before and during drug application are caused by voltage ramps. Figure 4.1b shows the current against voltage relationship constructed by subtracting the average of the voltage ramps performed before and during agonist application.
**Figure 4.2** shows the current against voltage relationship for one cell in the presence of 0μM, 50μM and 100μM Mg²⁺. I-V relationships were obtained by performing voltage ramps generated by software provided by Prof. D. Colquhoun (CJUMP4). Four voltage ramp trials were carried out before during and after drug application and each set averaged. The net current against voltage plot was constructed by subtracting the drug average from the averages of the trials before and after drug application. Each ramp protocol consisted of a 3 second ramp from -60mV to +40mV, then to -100mV and back to -60mV.
the region of 4µM (Gibb & Colquhoun, 1992). This was assumed to be the correction for contaminant Mg\(^{2+}\) in all the calculations.

Direct fitting of current voltage relations from control recordings gave a mean \(\delta\) of 0.920 ± 0.061 (n=14). There was a tendency for the \(\delta\) value estimated from direct fitting to increase with Mg\(^{2+}\) concentration but this was not statistically significant. Estimates of \(K_{Mg}(0)\) were more variable but the mean during control was 4.5 ± 2.6mM (n=14). This corresponds to a \(K_{Mg}(-60mV)\) of 0.057mM.

The magnitude of the inward current at -50mV in each Mg\(^{2+}\) concentration were compared to each other in relation to the current in 0mM Mg\(^{2+}\) to compare the relative reduction in current due to Mg\(^{2+}\) block. **Figure 4.3** shows the average relative current during agonist application in the presence of different \([Mg^{2+}]_{\text{Ext}}\) against the time of application of the agonist. As expected the relative current is attenuated to a greater degree in progressively higher \([Mg^{2+}]_{\text{Ext}}\). The outward current at positive membrane potentials was only slightly decreased at high \([Mg^{2+}]\) (**Figure 4.4**).

**4.3.2 Application of 1S, 3R ACPD to the slice**

As with the single channel cell-attached patch experiments, mGluRs were activated by bath application of 50µM ACPD for a minimum of 10 minutes. The average size of the current to 20µM NMDA and 5µM glycine after mGluR activation from cells clamped at -60mV was 12.5 ± 3.1pA (n=9). **Figure 4.5a**
Figure 4.3 shows relative current at -50mV relative to control current (in 0µM Mg$^{2+}$) against the time after start of experiment that agonist was applied (n=3). The blue plot shows response to 20µM NMDA and 5µM glycine during control periods and the red plot shows responses after mGluR activation. The period during which ACPD is applied is marked with the red line. As expected there is a clear progressive increase in attenuation of the current with increasing [Mg$^{2+}$] during control. The response in 0µM Mg$^{2+}$ during ACPD application is attenuated (in comparison to the control 0µM response). The successive agonist responses in increasing Mg$^{2+}$ do not show a large attenuation in comparison to the control responses.
Figure 4.4 shows responses normalised to 0μM Mg\(^{2+}\) at +20mV in the presence of progressively increasing [Mg\(^{2+}\)]. During control recording there is quite a large spread in the size of the current and unlike at negative potentials (Figure 4.3), the current at positive potentials does not show a marked attenuation in the presence of increasing [Mg\(^{2+}\)]. After application of ACPD the responses do not have such a large spread as during control and the [Mg\(^{2+}\)] sensitivity of the current at positive potentials is more obvious. The outward current is also attenuated at all potentials compared to the control recordings.
shows a representative whole-cell perforated patch response to 20μM NMDA and 5μM glycine after ACPD application. The current against voltage curve for this response are shown in Figure 4.5b. mGluR activation attenuated the NMDA current at all potentials in comparison to control application of NMDA.

NMDA and glycine were then applied in the presence of 50μM and then 100μM Mg^{2+} and current against voltage curves constructed. The δ value for magnesium block after mGluR activation obtained from direct fitting was 1.051 ± 0.124 (n=10) and the $K_{\text{Mg}(0)}$ value was 5.13 ± 1.56mM (n=3). This corresponds to a $K_{\text{Mg}(-60\text{mV})}$ of 0.035mM. These results suggest that mGluR activation does not significantly change the sensitivity of the NMDA channel to Mg^{2+} block.

4.3.3 Cyclosporin A pre-treatment of the slice

In one a separate experiment cyclosporin A (CsA) was applied to the slice in order to inhibit calcineurin prior to application of ACPD. Again the same experimental protocol was used, applying NMDA and glycine in the presence of different Mg^{2+} concentrations, followed by activation of mGluRs and then application of agonist. In this case the δ value for Mg^{2+} blockade obtained from direct fitting during control applications was 0.968 ± 0.065 (n=3) and the value of $K_{\text{Mg}(0)}$ was 2.28 ± 1.38mM (n=3). The current at -50mV in nominally 0μM Mg^{2+} was -9.5pA and at +20mV was +25pA. After ACPD application the δ value was 0.897 ± 0.026 (n=3), the value of $K_{\text{Mg}(0)}$ was 5.043mM (n=3) and the current at -50mV was -22pA and at +20mV was +7.2pA.
Figure 4.5a shows a whole cell response to 20 μM NMDA and 5μM glycine applied in the bath perfusate. The cell is voltage clamped at -60mV using the perforated whole cell recording configuration. 1mM Ba$^{2+}$ is included in the external solution. The deflections seen during drug application are caused by the voltage ramp protocol. Figure 4.5b shows the current against voltage relationship constructed by subtracting the average of the voltage ramps during control from the average of the voltage ramps during agonist application.
4.3.4 Divalent permeability

Voltage ramps (section 2.5) were performed on perforated patch whole cell responses to bath applied 20μM NMDA and 5μM glycine in the presence of 0.5mM and 5mM Ba$^{2+}$ in the external solution. ACPD was applied to the slice for a minimum of ten minutes and voltage ramps again performed during application of NMDA and glycine. The average reversal potential during control recording in 0.5mM Ba$^{2+}$ ($E_1$) was $+1.30 \pm 4.00$ mV (n=3) and in 5mM external Ba$^{2+}$ the average current reversal potential ($E_2$) was $+21.7 \pm 3.91$ mV (n=3). After application of ACPD the average current reversal potential in 0.5mM Ba$^{2+}$ ($E_3$) was $+11.3 \pm 3.27$ mV (n=3) and in 5mM Ba$^{2+}$ ($E_4$) was $+14.5 \pm 0.546$ mV (n=3). The average shift in $E_{rev}$ during control ($E_1-E_2$) and after ACPD ($E_3-E_4$) were calculated and substituted into Equation 4.2 (Section 4.2.2). The permeability $P_{Ba^{2+}}/P_{M^{+}}$ during control recording was 62.7 and after ACPD application 2.54.
4.4 Discussion

4.4.1 Mg$^{2+}$ sensitivity of the NMDA receptor-channel

Mg$^{2+}$ blockade of NMDARs is a crucial mechanism of control of neuronal excitability and synaptic integration. Modulation of this mechanism could therefore play an important role in many physiological and pathological processes. Chen & Huang (1992) reported that potentiation of NMDA-mediated responses in isolated rat trigeminal neurones when PKC was applied intracellularly was due to a reduction in Mg$^{2+}$ sensitivity.

However a study by Wagner & Leonard (1996) showed that PKC could only potentiate some heteromeric recombinant mouse NMDARs. They found that the dimer combinations ζ1/ε1 (NR1/2A) and ζ1/ε2 (NR1/2B) underwent PKC potentiation and ζ1/ε3 (NR1/2C) and ζ1/ε4 (NR1/2D) did not show any potentiation. The Mg$^{2+}$ sensitivity of the potentiated subunits is reduced by only a fraction of the change reported by Chen & Huang and does not account for the size of the potentiation seen. Wagner & Leonard (1996) concluded that PKC potentiation of the current is due to mechanisms other than a reduction in Mg$^{2+}$ sensitivity. The modulation of Mg$^{2+}$ sensitivity of the receptor by PKC activation may therefore not be a ubiquitous theme in the CNS but could be an important mechanism in some areas.
Inhibition of PKC activity or phosphatase activation might be expected to have the reverse effect to PKC activation i.e. an increase in the Mg\(^{2+}\) sensitivity of the receptor. There has been no direct evidence published to indicate that this is the case. However the action of phosphatase inhibitors causes an increase in the mean open time of the receptor (Lieberman & Mody, 1994; Wang et al., 1994). These reported changes in open time taken together with the PKC study of Chen & Huang (1992) and the results reported in Chapter 3 of this thesis suggest mGluRs could affect the NMDAR Mg\(^{2+}\) sensitivity through the action of phosphatases.

4.4.2 Is the modulation of NMDARs by ACPD due to a change in their Mg\(^{2+}\) sensitivity?

Initial perforated whole-cell experiments (discussed in more detail in Chapter 5) showed that mGluR activation could attenuate the whole-cell response. It was expected that the single channel current underlying the macroscopic current would be modulated by mGluRs in the same manner as that characterised in Chapter 3. As found in cell-attached patch recordings, the mGluR mediated modulation of the macroscopic current could be occluded by pre-treatment of the slice with CsA further reinforcing the idea that this modulation had the same underlying mechanism. In the light of previous work a reasonable hypothesis to test was for changes in NMDARs’ Mg\(^{2+}\) sensitivity using voltage ramps before and after mGluR activation.
Voltage ramps during perforated whole-cell patch recordings of NMDA responses should not greatly disrupt the normal physiology by avoiding washing out the contents of the cell and also the amphotericin pores created in the membrane patch prevent divalent buffering in the cytoplasm (Horn & Marty, 1988).

The Mg$^{2+}$ block was analysed using the simple sequential open channel blocking model (Neher & Steinbach, 1978) as a first approximation. The parameters obtained from the fit of the voltage ramps in each concentration of Mg$^{2+}$ were used to find a value for $\delta$ before and after mGluR activation. If the voltage sensitivity of Mg$^{2+}$ block is increased by mGluRs, as we had suspected, the value of $\delta$ would be expected to increase after mGluR activation while if the absolute affinity of Mg$^{2+}$ for its binding site in the channel were changed, the estimate of $K_{Mg^{2+}}(0mV)$ should change.

The values of $\delta$ obtained from direct fitting during control recording are similar to previous studies in which the $\delta$ values were in the region of 0.8-1 (Ascher & Nowak, 1988; Jahr & Stevens, 1990). The value of $\delta$ after mGluR activation showed no appreciable change to that during control, suggesting there was no change in voltage dependency. Similarly the value of $K_{Mg}(0mV)$ showed no difference after mGluR activation again suggesting that the mGluR mediated modulation of the NMDAR response did not manifest itself through a change in the Mg$^{2+}$ sensitivity of the channel.
As expected the inward current measured at -50mV was attenuated by increasing Mg\(^{2+}\) concentration (Figure 4.4) however after mGluR activation, the current showed a more marked attenuation when agonist was applied in nominally Mg\(^{2+}\) free external solution in comparison to the suppression in Mg\(^{2+}\). The large difference between the size of the current in zero Mg\(^{2+}\) before and after ACPD could be attributed to an increase in Mg\(^{2+}\) sensitivity (as there is residual Mg\(^{2+}\) contamination present). However, for the reasons stated above, it seems that this is unlikely and that the attenuation of the current is due to some other mechanism.

4.4.3 Divalent permeability of the NMDA receptor-channel

Divalent cation permeation of NMDA channels underlies part of their physiological importance in the CNS. Under normal conditions approximately 10% of the total current is carried by Ca\(^{2+}\) ions (Schneggenburger et al., 1993; Burnashev et al., 1995; Schneggenburger, 1996). This relatively high Ca\(^{2+}\) permeability is responsible for many of the patho-physiological processes which these receptors are associated with. In addition to simple permeation of the channel, recent evidence points to a high affinity binding site for Ca\(^{2+}\) near the external mouth of the channel pore (Premkumar & Auerbach, 1996). Ca\(^{2+}\) ions bind to this site and can reduce the sodium (Na\(^{+}\)) current.
4.4.4 Is the modulation of NMDA receptor-channels due to a change in their divalent permeability?

Since investigation of Mg\(^{2+}\) block of the receptor showed no change on ACPD application, we tested the idea that mGluR mediated modulation of the NMDAR-channel was due to an altered divalent cation permeability.

Voltage ramps performed on responses to NMDA in the presence of different Ba\(^{2+}\) concentrations were used to construct current against voltage plots. The shift in the \(E_{\text{Rev}}\) in different external Ba\(^{2+}\) concentrations showed an obvious decrease after ACPD application. The calculated permeability before and after ACPD were also significantly different. However the absolute value for the permeability during control recordings seems unrealistically high. Previous estimates of \(P_{\text{Ca}^{2+}}/P_{\text{M}^{+}}\) for NMDARs are in the range from 5 to 10 (Mayer & Westbrook, 1987; Forsythe & Westbrook, 1988; Jahr & Stevens 1993; Schneggenburger, 1996).

It is not possible from these results to conclude that there is a change in the divalent permeability of the NMDAR-channel after mGluR activation and further experiments using a larger range of Ba\(^{2+}\) concentrations are needed before any conclusions can be drawn about the permeability ratio obtained here.
Chapter 5  Modulation of NMDA-mediated whole-cell current responses by activation of metabotropic glutamate receptors

5.1 Summary

1. Whole-cell currents evoked by exogenous application of the agonists \( N \)-methyl-D-aspartate (NMDA) and glutamate were recorded from granule cells voltage clamped at -60mV in the dentate gyrus of the rat hippocampus.

2. Experiments designed to look for mGluR mediated modulation of the size of the NMDA current gave variable results. Co-application of an mGluR antagonist with glutamate produced potentiation in the size of the NMDAR current in some cases.

3. The sizes of whole-cell currents elicited by application of NMDA and glycine to these cells were measured before and after mGluR activation using the specific mGluR agonist ACPD. Again results were not consistent; in two recordings there was an average decrease of 33.7% in the size of the current however in two different recordings there was a clear potentiation averaging 55.0% observed after mGluR application.

4. In order not to wash-out the intracellular constituents of the cell, which may be involved in the mGluR transduction mechanism, the perforated whole-cell configuration of the patch-clamp technique was adopted. Also, external Ca\(^{2+}\)
was replaced by 1mM Ba\(^{2+}\) to prevent a rise in intracellular \([Ca^{2+}]_i\) due to influx through NMDA channels which might affect intracellular messenger systems. Under these conditions mGluR activation produced a clear attenuation of 29.9 ± 8.9% (n=9) in the whole-cell current responses to NMDA.

5. Stationary noise analysis gave an estimated single channel conductance of 37.1 ± 6.08pS (n=3) during control recording. When responses were compared directly in cells in which recordings were made before and after mGluR activation there was an apparent reduction in estimated single channel conductance of 18.0 ± 6.51% to 30.1 ± 4.26pS (n=3) after application of ACPD.
5.2 Introduction

Initial disappointment with cell-attached single channel recordings was puzzling (Section 3.3) and so to discount the possibility that NMDA channels were distributed in very low densities on the somatic membrane of these cells, whole-cell patch-clamp experiments applying exogenous agonist were performed.

When it was found that agonist application could elicit large and reproducible responses from these cells, further experiments were performed using agonists and antagonists of mGluRs to look for modulation of these whole-cell responses.

Although modulation of the whole-cell current was seen in a number of experiments these were not conclusive as sometimes mGluRs had no significant effect on the NMDA mediated current.

After further experiments had shown that mGluRs mediated a robust modulation of NMDA single channels (Chapter 3) further studies were performed to test if this would also be seen in the whole-cell current by employing different recording conditions.

Standard whole-cell recording techniques cause wash-out of the intracellular constituents of the cell. It was reasoned from the single channel data that it was probably important to keep these mechanisms intact for modulation to occur.
To obviate the washout problem, the perforated whole-cell technique, adapted from Horn & Marty (1988) and Rae et al. (1991), employing the use of the macrolide antibiotic amphotericin B as a pore former (see Section 2.2.4) was adopted. This enabled us to make whole-cell recordings without disrupting the intracellular constituents which could potentially be responsible for NMDAR modulation.

From the single channel results presented in Chapter 3 it was expected that mGluR activation would cause an attenuation of the whole-cell NMDA response. Kelso et al., (1992) showed that responses to NMDA from receptors expressed in oocytes were potentiated by activation of mGluRs by a mechanism involving PKC.

For native receptors, the intracellular mechanisms and G-proteins which couple to mGluRs might differ to those present in Xenopus oocytes. Therefore it would not be unreasonable to expect activation of mGluRs leading to phosphatase activation (via a rise in intracellular Ca^{2+}) and causing dephosphorylation (rather than phosphorylation) of the NMDAR which might result in an attenuation of the current.

In order to analyse the modulation further, we used the technique of noise analysis to provide estimates of the single channel conductance to see whether this would corroborate the reduction in slope conductance previously shown in single channel recordings (Section 3.3.2).
Whole-cell currents elicited by application of exogenous agonist are not a very subtle or high resolution method for studying channel behaviour. However use of the perforated whole-cell configuration provided an experiment where the environment of the receptors involved is likely to be close to normal.
5.3 Results

5.3.1 Conventional whole-cell configuration

Initial whole-cell experiments involved application of the agonists glutamate and NMDA (along with glycine) via a flow pipe placed approximately 100μm from the soma of the granule cells. Both agonists elicited large responses to relatively small concentrations of agonist. 10μM NMDA plus 3μM glycine produced an average inward of 275 ± 41.9 pA (n=10) from granule cells clamped at -60mV (the external solution contained 2mM Ca^{2+}). 10 μM glutamate plus 3μM glycine evoked a slightly larger average current of 341 ± 73.0 pA (n=9) (see Figure 5.1) as expected from previously published dose response curves for NMDA receptor activation in cultured neurones (Patneau & Mayer, 1990; Traynelis & Cull-Candy, 1991).

5.3.2 mGluR antagonism using L-AP3

In the first set of experiments testing the action of mGluRs, the mGluR selective antagonist L(+)-2-amino-3-phosphonopropionic acid (L-AP3) (Schoepp et al., 1990) was co-applied simultaneously with 1μM glutamate and 3μM glycine. Since, obviously, glutamate itself is a potent agonist of mGluRs, its application to the slice would cause activation of these receptors too. Therefore co-application of L-AP3 should produce information about the effect of mGluR activation on responses to glutamate.
Figure 5.1 shows representative whole cell responses to 10μM glutamate and 3μM glycine (red bar) and to 10μM NMDA and 3μM glycine (blue bar). Both agonists are applied via a flow pipe placed approximately 100μm away from the cell soma. The external Krebs solution contained 2mM Ca^{2+} and the cells were clamped at -60mV. The bar chart shows the average peak current elicited from 9 cells for glutamate (red fill) and from 10 cells for NMDA application (blue fill) under these conditions.
In these experiments 1µM glutamate and 3µM glycine applied using flow pipes elicited a current of 202.5 ± 70.4pA (n=4). Inclusion of 50µM L-AP3 into the agonist solution produced responses of 365.4 ± 100.3pA (n=4). Again these results were obtained with 2mM Ca^{2+} in the external solution. This suggests there is a large increase in the current when mGluRs are antagonised of +80.4%. However direct comparison of cells in which agonist was applied before and after antagonism of mGluRs (Figure 5.2) allows the average percentage change in size of the response of +14.4 ± 34.8 % (n=3) to be calculated.

5.3.3 Activation of mGluRs with 1S, 3R ACPD

NMDA was also used as an agonist and modulation of the whole-cell current by 50µM 1S, 3R ACPD was tested. In these experiments 1mM Ca^{2+} was included in the external solution along with 100nM tetrodotoxin (TTX) and 10µM bicuculline. Drug applications were made using a flow pipe situated near the cell soma. 10µM NMDA and 3µM glycine elicited responses of 150.0 ± 27.2 pA (n=4). Application of 50µM ACPD for approximately 3 minutes before and also during NMDA application gave a NMDA elicited response of 113.4 ± 27.2pA (n=4).

Although these results suggest that mGluR activation attenuated NMDA mediated responses, the results are not so conclusive if the individual cells are considered (see Figure 5.3). For two of the four cells there was a large attenuation in the size
Figure 5.2a shows representative whole cell responses from one cell induced by application of 1μM glutamate and 3μM glycine (red bar) or by glutamate and glycine with 50μM L-AP3 (green bar). Agonist applications are made with a flow-pipe situated near to the cell soma. External solution contains 2mM Ca$^{2+}$. The bar chart shows the average current from three cells again to 1μM glutamate and 3μM glycine (red fill) or to glutamate, glycine and 50μM L-AP3 (green fill).
Figure 5.3 shows peak whole cell responses of four cells to 10μM NMDA and 3μM glycine applied via a flow pipe near the cell soma before (red fill) and after (green fill) application of 50μM ACPD to the slice. It is obvious that there is no pattern to the responses after application of ACPD with both potentiation and attenuation of the NMDA response being seen. The average change in the response is $+10 \pm 30.2\%$ ($n=4$). One possibility for this variability could be washout of the cell interior during whole cell recording.
of the whole-cell response of 33.8% and responses of the other two cells showed a large potentiation of 55%. Thus comparing the size of the response after ACPD application to the size before for each particular cell, there is an average percentage increase in the size of the response of +10.6 ± 30.2% (n=4).

5.3.4 Activation of mGluRs using the perforated whole-cell configuration

Experiments employing the whole-cell configuration gave variable results. Since the mechanism of mGluR modulation of NMDARs may be dependant on intracellular enzymes (see Section 3.4.2), the inconsistencies in the results could have occurred due to washout of the cell interior. In order to obviate the washout problem the perforated whole-cell configuration was adopted. The external Ca$^{2+}$ was also replaced by Ba$^{2+}$. This was to prevent rises in [Ca$^{2+}$]$_i$ on activation of NMDARs affecting intracellular signalling pathways. Cells were again voltage clamped at -60mV and all agonists were bath applied. 300nM TTX was included in the bathing solution in some experiments.

The average size of the current when 20μM NMDA and 5μM glycine were bath applied to the slice was 17.3 ± 3.2pA (n=9). The same agonists after 50μM ACPD had been applied to the slice for a minimum of 10 minutes produced an average response of 12.5 ± 3.1pA (n=9) (see Figure 5.4). Comparing responses of individual cells before and after ACPD, there is a consistent attenuation in the NMDA mediated response -29.9 ± 8.9% (n=9) after application of ACPD.
Figure 5.4 shows the average size of peak whole cell perforated patch responses from granule cells voltage clamped at -60mV. 20μM NMDA and 5μM glycine were bath applied to hippocampal slices. The external solution contained 1mM Ba^{2+}. Applications were averaged between nine cells before, red fill (control), and after, blue fill (ACPD), application of 50μM ACPD. The average percentage reduction in the size of the response after mGluR activation is 30 ± 8.9% (n=9).
The consistent nature of the small size of the NMDA response in these perforated whole-cell patch recordings in comparison to the relatively large currents induced in the whole-cell configuration could have been due to the difference in the method of agonist application. However this alone seems unlikely to cause such a large difference and another suggestion is that this difference is analogous to the small amount of single channel activity in cell-attached patches compared to outside-out patches.

5.3.5 Effect of activation of mGluRs on AMPA mediated current

In one perforated patch recording 10μM AMPA was applied to a granule cell clamped at -60mV. mGluRs were activated by applying 50μM ACPD for ten minutes and then AMPA was again applied to the cell. The average size of the response to three control applications of AMPA to the same cell was 38.3 ± 1.43pA. After mGluR activation the average size of the response to three AMPA applications again to the same cell was 40.5 ± 0.62pA (see Figure 5.5).

5.3.6 Using fluoride to inhibit intracellular phosphatases

In these experiments perforated whole-cell recordings were made with a pipette solution containing CsF (see Section 2.2). The amphotericin pores are permeable to fluoride (F⁻) which permeates into the cell and is known to block phosphatase activity (Cohen, 1989). Using this solution the average size of the current to bath applied 20μM NMDA and 3μM glycine was 36.3 ± 20.5 pA (n=3) and after
Figure 5.5 shows two applications of AMPA to a granule cell from the hippocampus of a P14 rat. The cell is voltage clamped at -60mV using the perforated whole cell configuration. Bath application of 10μM AMPA elicited a reproducible current of 38.3 ± 1.43pA (three applications to the same cell). After mGluRs had been activated by applying 50μM ACPD to the slice 10μM AMPA elicited a response which showed no modulation in size (40.5 ± 0.623pA).
ACPD 53.0 ± 25.7 pA (n=3). 1mM Ca\(^{2+}\) was present in the external solution. The average percentage change in current shows an increase of +30.5 ± 42.5%. In one of these recordings the PKC inhibitor calphostin C was applied for approximately 5 minutes prior to ACPD. The size of the current was initially 39pA and after calphostin C and ACPD application the current was 32pA.

5.3.7 Noise analysis

In three cells noise analysis was used to allow comparison of the power spectra before and after mGluR activation (shown in Figure 5.6) to test whether any changes in channel conductance would be detected using this method of analysis. The perforated whole-cell recording configuration was used with an extracellular solution containing 1mM Ba\(^{2+}\). During control recording the average single channel conductance calculated using this method was 37.1 ± 6.1pS (n=3) and after mGluR activation was 30.1 ± 4.3pS (n=3). The average percentage change calculated for pairs of responses from the same cells before and after mGluR application shows a fairly large decrease in single channel conductance of 18.0 ± 6.5 % (n=3).
Figure 5.6 shows two representative power spectra from whole cell responses to 20μM NMDA and 5μM glycin e. In a. and b. the background spectrum is shown by x and the net drug-induced spectrum is shown by +. Spectra a. is produced from control recording whereas spectra b. is produced after ACPD application. Both spectra are fit by two Lorentzian equations. The single channel conductance calculated from spectra a. is 49.2pS and from spectra b. is 37.4pS.
5.4 Discussion

Initial experiments using conventional whole-cell recording techniques gave variable results with no reproducible, robust modulation of the NMDA response being observed. In some cells there seemed to be an attenuation in the NMDA response after mGluR activation and in others there was a potentiation in the size of the response.

Results presented in Chapter 3 showing a robust attenuation of single channel currents due to mGluR activation had suggested that the whole-cell current would show a similar modulation. This was not consistently observed and in fact the average percentage change in the current after ACPD application for individual cells showed an increase of $+10.6 \pm 30.2\%$ (n=4).

The single channel experiments in Chapter 3 made use of the cell-attached configuration. This technique kept the intracellular constituents of the cell intact and probably these are involved in the modulation of NMDARs by mGluR activation.

The perforated whole-cell technique allows the cell to be voltage clamped but avoids washout of the intracellular contents. Electrical access to the cell is impaired with typical series resistance measurement in the range of 30-60 MΩ. However since the size of the NMDA current was small in these experiments the series resistance error would be small.
The extracellular Ca\(^{2+}\) was also replaced by Ba\(^{2+}\) in these experiments. Since the NMDAR-channel is highly permeable to Ca\(^{2+}\) (Mayer & Westbrook, 1987; Schneggenburger, 1996), activating the receptor causes an increase in \([\text{Ca}^{2+}]_i\), which could potentially disrupt a number of Ca\(^{2+}\) dependant mechanisms. mGluR activation when recording using these procedures produced a clear attenuation in the NMDA response.

In recordings from three cells we took advantage of the fact that the membrane pores formed by amphotericin, although impermeable to large ions and molecules, allow small monovalents to pass into the cell. Therefore a pipette solution containing a high concentration of F\(^-\) would lead to permeation of F\(^-\) into the cell down it’s concentration gradient. F\(^-\) is a non-specific phosphatase inhibitor (Cohen, 1989). The results of these experiments although not conclusive, showed an increase in the size of the response after application of ACPD. This seemed rather strange as, in the light of what has previously been said, we would have expected inhibition of phosphatases to manifest itself by occluding the expected mGluR attenuation of the NMDA response. There are a number of possible explanations for the observed potentiation which need further investigation.

Firstly the inhibition of phosphatases due to F\(^-\) may result in mGluR activation causing potentiation rather than attenuation of responses because one side of the two counter balancing pathways has been blocked. This mechanism of potentiation could be due to PKC activation since mGluR activation following
application of the PKC inhibitor calphostin C produced no change of the NMDA response (in the one cell tested). This mechanism of modulation could also explain results from other studies showing mGluR mediated potentiation of responses in receptors expressed in *Xenopus* oocytes (Kelso *et al.*, 1992) if the oocyte biochemistry happens to be dominated by PKC.

Secondly the F' mediated inhibition of phosphatases could have uncovered a basal intrinsic inhibition of NMDA receptors by phosphatases. This possibility suggests a tonic inhibitory phosphorylation of NMDA receptors. However this interpretation is complicated by the possibility that kinases such as CaMKII may be regulated by phosphorylation/dephosphorylation reactions.

Or thirdly the activation of NMDA receptors when phosphatases are inhibited could be responsible for a rise in \([Ca]_i\) (due to influx through the NMDAR-channel) which could activate kinases causing a potentiation in the response which is normally inhibited to some degree through mGluR activation. The mechanism could be tested by repeating the experiments with F' when extracellular Ca\(^{2+}\) is replaced by Ba\(^{2+}\).

Stationary variance analysis performed on a number of recordings estimated the single channel current from perforated whole-cell responses in the presence of extracellular Ba\(^{2+}\) before and after mGluR application. Three recordings were analysed in which responses to three separate applications of 20µM NMDA and 5µM glycine were made before and after mGluR activation in the same cell. There
was quite a large spread of conductances which masked to some extent the individual results which are collated in Table 4. Noise analysis can sometimes produce anomalous results and on its own this reduction in single channel conductance should be judged critically, however in the light of the single channel data presented in Chapter 3 this result may be used to tentatively suggest that the single channel conductance of somatic NMDA channels is reduced by mGluR activation and that would contribute to a reduction in the size of the whole-cell current.

Table 4 shows the single channel conductance (in pS) measurements calculated using noise analysis for three cells. The percentage change in the estimated single channel conductance is shown. All analysis was performed on responses to 20μM NMDA and 5μM glycine from granule cells in the dentate gyrus. Ca^{2+} was substituted with 1mM Ba^{2+} in the external solution.

<table>
<thead>
<tr>
<th>Cell no</th>
<th>Control</th>
<th>ACPD</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>30.3</td>
<td>-4.95</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>Standard Error</td>
<td>6.08</td>
<td>4.26</td>
<td>6.51</td>
</tr>
</tbody>
</table>
Chapter 6 Discussion of the thesis

This thesis deals with cross talk between two glutamate activated receptors. The experimental results illustrate the phenomenon whereby the activation of mGluRs, which are co-localised with NMDARs on the postsynaptic membrane (Baude et al., 1993), modulate the opening behaviour of the ionotropic NMDARs. Experiments described in Chapter 3 compared the NMDAR single channel properties before and after mGluR activation. These studies have produced surprising results as in addition to attenuation of open periods, bursts and cluster lengths of the receptor (which have been reported previously in studies looking at the effect of phosphatase inhibitors (Lieberman & Mody 1994; Wang et al., 1994)), there was a distinct reduction in the NMDAR-channel slope conductance.

6.1 What is the mechanism of mGluR mediated modulation of NMDARs?

Previous studies looking at receptor modulation have suggested that NMDARs can be functionally modulated by processes which phosphorylate or dephosphorylate the receptor protein. As mGluRs activate many of the intracellular pathways which lead to activation of kinases and phosphatases this was an obvious area to study when looking for the mechanism of mGluR mediated modulation.
Inhibition of calcineurin, using the immunosuppressant CsA, points to this phosphatase being involved in the modulation of NMDARs. Previous reports have implicated this enzyme in the modulation of NMDAR-channel open times (Liebermann & Mody, 1994) and NMDAR desensitisation (Tong et al., 1995). It has also been shown to modulate GABA_A receptors (Glaum & Miller, 1994). Results presented in Chapter 3 would suggest that calcineurin is activated by mGluRs which causes a dephosphorylation of the receptor (which must be basally phosphorylated) leading to an attenuation in the receptor’s single channel properties.

Activation of Group I mGluRs is likely to cause a rise in intracellular Ca^{2+} and activation of PKC. Because manipulation of intracellular Ca^{2+} will affect PKC as well as calcineurin the effects of changing Ca^{2+} are not clear cut. Initial results using an inhibitor of PKC complicate the interpretation as it seems that inhibition of this enzyme also partially inhibits the modulatory action of mGluRs. In Section 3.3.7 inhibition of PKC occludes the reduction in slope conductance but not the attenuation in the NMDAR-channel open times, burst and cluster lengths caused by mGluR activation. One problem when considering these intracellular mechanism is that many of these enzymes have multiple effects and so it is difficult to say for certain that using an inhibitor will provide a clear result. For example, inhibition of calcineurin could result in an up-regulation of another phosphatase or kinase.
A suggestion of a co-dependence between different intracellular systems was also seen in results presented in Chapter 5 (Section 5.3.5) where inhibition of phosphatases with intracellular F' surprisingly produced an increase in the NMDAR response after mGluR activation. Inhibition of PKC in this instance occluded this potentiation suggesting that mGluRs, in the absence of phosphatase activity, may activate PKC to potentiate the NMDAR response (Chen & Huang, 1992). Another possible explanation for these interesting results is that mGluR function itself might be altered by using enzyme inhibitors perhaps directly or by promoting mGluR desensitisation (Gereau & Heinemann, 1996) (see Section 1.4.2).

From the results discussed in this thesis and from previously published studies it seems likely that there is a balance between phosphorylation and dephosphorylation of NMDARs which can be influenced by, among other things, mGluR activation, the level of phosphorylation of the NMDAR having a large influence on the functional behaviour of the receptor.

6.2 How does the modulation affect the function of the receptor?

Previous studies by Chen & Huang (1992), had suggested that activation of PKC caused a reduction in the NMDAR Mg\(^{2+}\) sensitivity and hence a potentiation of the NMDA mediated response. Based on this, an attractive hypothesis for the mGluR mediated modulation of NMDARs might be a dephosphorylation of the receptor by calcineurin causing an increase in the Mg\(^{2+}\) sensitivity leading to an
attenuation of the NMDAR response. However in Chapter 4, when this hypothesis was tested by comparing the values of $\delta$ and $K_{Mg^{2+}}$ before and after mGluR activation, our results suggested that mGluRs have no significant effect on Mg$^{2+}$ sensitivity of the NMDAR.

Another possible way in which the modulation might manifest itself is through a conformational change affecting the receptors divalent permeability. In Section 4.3.4 this hypothesis was tested by comparing the current reversal potential in various $[Ba^{2+}]_{ext}$. The data showed that there is indeed a difference in the $E_{rev}$ shift before and after ACPD, and there is a large difference in the calculated permeability of the receptor before and after application of ACPD. However the average divalent permeability of the receptor derived from control recording seems unrealistically high. Therefore, although it remains a possibility that the divalent permeability of the receptor is reduced by mGluRs, further experiments are needed to test this.

6.3 Consequences for synapse physiology

It would be interesting to see whether this mGluR mediated NMDAR modulation is active at the synapse. Any such modulation of NMDA response would of course have implications for understanding the role NMDARs play in synaptic transmission. It does not seem unreasonable that group I mGluRs, which have been shown to be situated on the peri-synaptic membrane of neurones (Baude et al., 1993) could act to reduce the NMDAR mediated synaptic current through an
intracellular mechanism involving the action of enzymes on the receptor protein (see Figure 6.1 for a hypothetical model of mGluR mediated modulation at the synapse). In fact a mechanism such as this would be an effective way of controlling the excitability of the cell during periods of increased neurotransmitter release. The reduction in single channel conductance of the NMDARs suggests that the peak amplitude of the NMDA mediated EPSC would be attenuated by mGluR modulation. Similarly the reduction in burst and cluster length of the single channels would suggest that the time-course of the synaptic current might also undergo attenuation by mGluR activation, depending on whether the channel 1st latencies are also affected. The attenuation in somatic receptor responses may also act as a neuroprotective device during ischaemic insult (Koh et al., 1991; Birrell et al., 1993) when levels of glutamate in the interstitial space could rise.

The ubiquitous distribution of both mGluRs and NMDARs suggests that mGluR mediated modulation of NMDARs could be an important mechanism in cellular function in the CNS. The specific and discrete patterns of expression in the CNS of the NR1 subunit splice variants (Laurie & Seeburg, 1994) and the NR2 subunits (Monyer et al., 1994) enable them to confer distinct functional properties on NMDARs in different brain areas and could potentially produce differences in the ability of the native receptors to be modulated. The results of this study might therefore further suggest that NMDAR and mGluR distributions need to be considered together in understanding the role of NMDARs during development and in the mature CNS.
Figure 6.1 shows a cartoon representation of a possible mechanism for the modulation of NMDARs by mGluRs. At the synapse, peri-synaptic mGluRs are activated by over-flow of glutamate during periods of high release. These activate intracellular mechanisms through phospholipase C (PLC) and inositol triphosphate (IP$_3$) which activate calcineurin through release of Ca$^{2+}$ from intracellular stores. Calcineurin dephosphorylates the receptor causing an attenuation in the receptors response. This would be a very convenient way of modulating postsynaptic receptors, causing a negative feedback to limit inappropriate receptor activation and could limit Ca$^{2+}$ influx into the cell which may have implication for a number of pathologies including anoxic neurotoxicity.
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