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An investigation of NMDA receptor subunit pharmacology

Andrew Peter Mallon

Institute of Biomedical and Life Sciences,
Division of Neuroscience and Biomedical Systems,
University of Glasgow.

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Thesis submitted in part fulfilment of the requirement for admission to the degree of Doctor of Philosophy of the University of Glasgow.
Abstract

N-Methyl-D-aspartate (NMDA) receptors are critically involved in synaptic transmission, neural development and various forms of neuronal plasticity including long-term potentiation (LTP) and long-term depression (LTD). They are also involved in the production of neuronal damage following excessive activation by glutamate released as a result of hypoxia or ischaemia. Each heteromeric receptor includes one or two NR1 subunits, at least two of the four NR2A-D subunits and less usually the NR3A/B subunits. This study demonstrates that the putative NR2B subunit-containing NMDA receptor antagonist Ro 25-6981 potentiates the effects of NMDA on rat hippocampal slices. The NR2A subunit antagonist PEAQX blocks the effects of NMDA alone and the potentiated response following Ro 25-6981 application. Furthermore, Ro 25-6981 was not neuroprotective as reported previously but unexpectedly precipitated excitotoxicity. The potentiating effect of Ro 25-6981 required around 20 minutes to become apparent, took a further 30 minutes to reach its maximum effect and was irreversible. It was not prevented by staurosporine (a broad-spectrum protein kinase inhibitor), okadaic acid (a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A) or anisomycin (a protein synthesis inhibitor). However, the potentiation was prevented by cyclosporin A (an inhibitor of Ca\(^{2+}\)/calmodulin-dependent phosphatase 2B [calcineurin]). The results
indicate that in an intact neuronal network, NR2B subunits tonically gate NR2A subunit-containing receptor function by a negative coupling mechanism involving calcineurin activation.

NMDA receptor-dependent LTP induced by high frequency stimulation was prevented by PEAQX, an NR2A antagonist. Ro 25-6981 was unable to prevent LTP induction but was associated with a marginal reduction in the magnitude of LTP induced.

There is evidence for the binding of homoquinolinic acid to an NMDA-insensitive novel binding site in the brain. This study investigated the pharmacology of homoquinolinate on the evoked field excitatory synaptic potential (fEPSP) recorded from the CA1 area of rat hippocampal slices. Two NMDA receptor agonists, quinolinic acid 150µM and homoquinolinic acid 2.5µM, caused an approximately 50% inhibition of fEPSP slope. Paired-pulse studies suggested there might be a presynaptic component to this action that is independent of presynaptic adenosine A₁ receptor activation. The broad-spectrum EAA antagonist kynurenic acid and the NMDA receptor blockers 2-amino-5-phosphonopentanoic acid and dizocilpine could prevent the inhibition of fEPSP slope. None of these antagonists revealed any other NMDA-insensitive activity of homoquinolinic acid. The use of 2-carboxy-3-carboxymethylquinoline (CCMQ) to displace the reported NMDA-insensitive binding had no effect on either baseline fEPSP slope or the depression caused by homoquinolinic
acid. It was also apparent that responses to homoquinolinic acid were blocked completely by the NR2A subunit-selective antagonist PEAQX, but not by the NR2B subunit-selective blocker Ro 25-6981. It was concluded that the novel binding site for homoquinolinic acid does not affect synaptic potentials in the hippocampus and that homoquinolinic acid appears to be a selective agonist at NMDA receptors that include the NR2A subunit. Although the NR2B agonist site may be maximally activated under normal conditions and therefore it is not possible to observe any additional effects upon fEPSP slope.

This study next investigated the negative coupling between NR2B and NR2A subunit-containing receptors, combining the NR2A/B subunit selective agonist HQA with the NR2B and NR2A selective antagonists Ro 25-6981 and PEAQX. The negative coupling observed previously with applications of NMDA was also seen using HQA and QA. The potentiation of responses to HQA by Ro 25-6981 application was also associated with an enhancement of paired-pulse interactions. The subsequent application of PEAQX was able to block both the depression of fEPSP slope and the associated enhancement of paired-pulse interactions. The presence of a presynaptic element during applications of HQA alone and potentiated responses alike and the blockade of these effects by PEAQX suggests the NR2A subunit-containing NMDA receptor is responsible for the presynaptic effects acting either directly at presynaptic
sites or indirectly at postsynaptic sites leading to the raising of a retrograde signal. The NR2B subunit in both its activated and antagonised state was associated with enhancements in paired-pulse interactions which suggest that it is not able to modulate directly the presynaptic element. However, whilst paired-pulse interactions are generally accepted to be presynaptic phenomena, it does not follow that postsynaptic effects cannot influence the appearance of changes in these interactions in field recordings. The absence of any observable difference between HQA, QA and NMDA results suggests that the NR2D subunit is not obviously involved in these processes.
Dedication

To my grandparents,

Peter and Agnes

Anthony and Agnes
Acknowledgements

I would like to extend my gratitude to Professor Trevor Stone and to Professor Hugh Nimmo for the opportunity to pursue research in their laboratories and for their help and expertise in realising my aims.

Special thanks go to the efforts made by the Bower Fire committee in their handling of this eponymous incident and the efforts made to prevent excessive ill effects to the course of my research.

I would also like to thank the many members of staff who provided me with help and advice.

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I would especially like to acknowledge the congenial company and assistance provided by the many members of the various laboratories I have researched in and frequented.

My fondest regards for my friends especially Ali, Annamieke, Chand, Chris, John, Kara, Leanne, Melissa, Sevil, Torfi and my girlfriend Julia.

In particular, I am indebted to my family, both immediate and extended, for their dependable love and support, especially Ben.
Contents

Abstract ........................................................................................................ 2
Dedication .................................................................................................... 6
Acknowledgements ...................................................................................... 7
Contents ........................................................................................................ 8
List of figures ............................................................................................. 12
List of tables ............................................................................................... 16
Abbreviations ............................................................................................ 17
Declaration ................................................................................................. 20
Publications ................................................................................................ 21

CHAPTER 1. INTRODUCTION ................................................................. 23
NMDA receptor: an historical perspective............................................. 23
NMDA receptor physiology ..................................................................... 24
NMDA receptor molecular biology ......................................................... 26
NMDA receptor distribution ................................................................... 28
NMDA receptor development ............................................................... 30
NMDA receptor pharmacology .............................................................. 34
AMPA and kainate receptors ................................................................. 37
Inhibitory GABA-ergic transmission ..................................................... 38
Anatomy of the hippocampus ............................................................... 42
Neuronal circuits in the hippocampus .................................................... 44

The hippocampal slice preparation ....................................................... 47

Extracellular responses ....................................................................... 51

Paired-pulse interactions .................................................................... 52
  Paired-pulse facilitation ................................................................. 52
  Paired-pulse depression ................................................................... 53

Aims ...................................................................................................... 56

CHAPTER 2. MATERIAL AND METHODS........................................ 57

Preparation of hippocampal slices ...................................................... 57

Composition of ACSF ......................................................................... 58

Bath superfusion and application of drugs .......................................... 58

Stimulation .......................................................................................... 59

Recording ............................................................................................. 61

Data analysis ......................................................................................... 62

Statistical analysis ................................................................................ 62

Chemical agents and drugs ................................................................. 63

CHAPTER 3. THE NEGATIVE COUPLING OF NR2B TO NR2A
SUBUNIT-CONTAINING RECEPTORS. .............................................. 65

Introduction ........................................................................................ 65

Results ................................................................................................ 72
  NMDA .............................................................................................. 72
  Ro 25-6981 ..................................................................................... 77
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMDA receptor antagonism</td>
<td>83</td>
</tr>
<tr>
<td>PEAQX</td>
<td>95</td>
</tr>
<tr>
<td>Protein phosphorylation</td>
<td>97</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>104</td>
</tr>
<tr>
<td>Discussion</td>
<td>106</td>
</tr>
<tr>
<td><strong>CHAPTER 4. THE NMDA SUBUNIT SPECIFICITY OF LTP</strong></td>
<td>112</td>
</tr>
<tr>
<td>Introduction</td>
<td>112</td>
</tr>
<tr>
<td>Results</td>
<td>115</td>
</tr>
<tr>
<td>Discussion</td>
<td>122</td>
</tr>
<tr>
<td><strong>CHAPTER 5. THE NR2A &amp; NR2B SUBUNIT-SELECTIVITY OF</strong></td>
<td>124</td>
</tr>
<tr>
<td>HOMOQUINOLINIC ACID</td>
<td>124</td>
</tr>
<tr>
<td>Introduction</td>
<td>124</td>
</tr>
<tr>
<td>Results</td>
<td>126</td>
</tr>
<tr>
<td>HQA and QA</td>
<td>126</td>
</tr>
<tr>
<td>DPCPX</td>
<td>137</td>
</tr>
<tr>
<td>Inhibition by kynurenic acid, 2-AP5 and dizocilpine</td>
<td>141</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>146</td>
</tr>
<tr>
<td>Excitotoxicity</td>
<td>150</td>
</tr>
<tr>
<td>NR2A antagonists</td>
<td>153</td>
</tr>
<tr>
<td>CCMQ</td>
<td>153</td>
</tr>
<tr>
<td>Zinc</td>
<td>158</td>
</tr>
<tr>
<td>PEAQX</td>
<td>160</td>
</tr>
<tr>
<td>PEAQX’s inhibition of the Ro 25-6981/HQA potentiation</td>
<td>165</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>168</td>
</tr>
<tr>
<td>AMPA and kainate receptors</td>
<td>170</td>
</tr>
</tbody>
</table>
List of figures

Figure 1. The NMDA receptor................................................................. 36
Figure 2. Diagram showing the anatomy of the rat hippocampal formation and the placement of the stimulating and recording electrodes............ 46
Figure 3. Example responses recorded from the CA1 region of the rat hippocampal slice................................................................. 60
Figure 4. Concentration curve of the inhibitory effect of NMDA upon fEPSP slope................................................................. 73
Figure 5. Effect of NMDA 10µM on fEPSP slope................................. 74
Figure 6. Effect of NMDA 10µM on paired-pulse interactions............... 75
Figure 7. Example records of paired-pulse interactions during baseline recording and under the influence of 10µM NMDA......................... 76
Figure 8. Repeated 10-minute applications of 10µM NMDA and the effect of 3µM Ro 25-6981................................................................. 80
Figure 9. Representative fEPSPs showing excitotoxic damage............... 81
Figure 10. Effect of Ro 25-6981 and NMDA 4µM on fEPSP slope......... 82
Figure 11. Effect of Ro 25-6981 on the continuous application of NMDA 4µM and its reversal by 2-AP5................................................................. 86
Figure 12. Effect of NMDA 4µM and Ro 25-6981 on paired-pulse interactions................................................................. 87
Figure 13. Effect of Ro 25-6981 on the continuous application of NMDA 7.5µM and its reversal by 2-AP5................................................................. 88
Figure 14. The concentration-response curve of NMDA alone and after Ro 25-6981................................................................. 89
Figure 15. Effect of 4µM NMDA and Ro 25-6981 in the presence of MK 801 on fEPSP slope................................................................. 91
Figure 16. Effect of 2-AP5 continuously bath-applied during the application of 4µM NMDA and Ro 25-6981 on fEPSP slope.......... 94
Figure 17. Effect of NMDA, Ro 25-6981 and PEAQX on fEPSP slope. 96
Figure 18. Action of staurosporine upon the effect of the co-application of
4μM NMDA and Ro 25-6981 on fEPSP slope. 100
Figure 19. Action of okadaic acid upon the effect of the co-application of
4μM NMDA and Ro 25-6981 on fEPSP slope. 101
Figure 20. Prevention by cyclosporin A of the effect of the co-application
of 4μM NMDA and Ro 25-6981 on fEPSP slope. 102
Figure 21. Column bar graph summary of phosphorylation modulators. 103
Figure 22. Effect of anisomycin on the Ro 25-6981/NMDA effect upon
fEPSP slope. 105
Figure 23. Effect of high frequency stimulation on fEPSP slope. 116
Figure 24. Representative fEPSPs demonstrating LTP. 117
Figure 25. Blockade of NMDA-dependent LTP induction by 2-AP5. 118
Figure 26. Blockade of NMDA-dependent LTP induction by PEAQX. 119
Figure 27. Effect of Ro 25-6981 on NMDA-dependent LTP induction. 120
Figure 28. Role of NR2A and NR2B subunits in long-term potentiation.
121
Figure 29. The concentration-response curve of HQA. 127
Figure 30. Effect of HQA 2.5μM on fEPSP slope. 128
Figure 31. Representative fEPSPs showing effect of 2.5μM HQA. 129
Figure 32. Effect of 2.5μM HQA on paired-pulse interactions. 130
Figure 33. Example records of fEPSPs during paired-pulse interactions.
131
Figure 34. The concentration-response curve of QA. 132
Figure 35. Effect of 150μM QA on fEPSP slope. 133
Figure 36. Representative fEPSPs showing the effect of QA 150μM. 134
Figure 37. Effect of QA on paired-pulse interactions. 135
Figure 38. Example records of fEPSPs during paired-pulse interactions.
136
Figure 39. Effect of DPCPX upon HQA-evoked depression of fEPSP slope. ................................................................................................... 139
Figure 40. Effect of HQA and DPCPX upon paired-pulse interactions. 140
Figure 41. Effect of kynurenic acid on QA and HQA-induced depressions of fEPSP slope ................................................................. 143
Figure 42. Effect of 2-AP5 on QA and HQA on fEPSP slope. ............. 144
Figure 43. Effect of MK 801 on QA and HQA on fEPSP slope. .......... 145
Figure 44. Effect of Ro 25-6981 with QA and HQA on fEPSP slope. ... 147
Figure 45. Effect of Ro 25-6981 and QA on paired-pulse interactions... 148
Figure 46. Effect of Ro 25-6981 and HQA on paired-pulse interactions. 149
Figure 47. Effect of HQA 4μM and Ro 25-6981 on fEPSP slope. ...... 151
Figure 48. Representative fEPSP recordings showing excitotoxicity .... 152
Figure 49. Effect of HQA 2.5μM and CCMQ 0.5mM on fEPSP slope. 155
Figure 50. Effect of CCMQ and Ro 25-6981 on fEPSP slope. ............ 156
Figure 51. Effect of HQA 2μM, Ro 25-6981 and CCMQ 0.5mM on fEPSP slope. ................................................................................................... 157
Figure 52. Effect of HQA 2.5μM and Zn 2+ 100nM on fEPSP slope. ...... 159
Figure 53. Effect of HQA and PEAQX on fEPSP slope. ..................... 162
Figure 54. The concentration-response curve of PEAQX on HQA-induced depression of fEPSP slope. ................................................................. 163
Figure 55. Effect of HQA and PEAQX on paired-pulse interactions. .... 164
Figure 56. Effect of HQA, Ro 25-6981 and PEAQX on fEPSP slope. ... 166
Figure 57. Effect of HQA, Ro 25-6981 and PEAQX on paired-pulse interactions. ......................................................................................... 167
Figure 58. Effect of HQA, Ro 25-6981 and anisomycin upon fEPSP slope. ............................................................................................................ 169
Figure 59. Effect of Ro 25-6981 and AMPA on fEPSP slope. ............ 171
Figure 60. Effect of Ro 25-6981 and KA on fEPSP slope ................. 172
Figure 61. Phosphatase hypothesis ...................................................... 194
Figure 62. Intracellular recordings of NMDA responses and the effect of Ro 25-6981 ................................................................. 198

Figure 63. Intracellular recordings showing the effect of NMDA alone and after the application of Ro 25-6981. ...................................................... 199
List of tables

Table 1. Summary of the distribution and developmental course of NMDA receptor subunits. ................................................................. 31
Table 2. Summary of the functional heterogeneity of NMDA receptors depending on the presence of specific NR2 subunits. ............... 32
Table 3. Summary of the general distribution of excitatory amino acid (EEA) receptors in the mammalian CNS......................................... 33
Table 4. List of pharmacological agents, suppliers and solvents used to produce stock solution. ............................................................. 64
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\theta_m$</td>
<td>modification threshold</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>2-AP5</td>
<td>D-2-amino-5-phosphono-pentanoic acid</td>
</tr>
<tr>
<td>BCM</td>
<td>Bienenstock-Cooper-Munro</td>
</tr>
<tr>
<td>CaMKII</td>
<td>$\mathrm{Ca}^{2+}$/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CCMQ</td>
<td>2-carboxy-3-carboxymethylquinoline</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CyA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPCPX</td>
<td>1,3-dipropyl-8-cyclopentyl-xanthine</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>50% excitatory concentration</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GlnBP</td>
<td>glutamine binding protein</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HFS</td>
<td>high frequency stimulation</td>
</tr>
<tr>
<td>HQA</td>
<td>homoquinolinic acid</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>KA</td>
<td>kainate</td>
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<td>KYA</td>
<td>kynurenic acid</td>
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<td>LFS</td>
<td>low frequency stimulation</td>
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<td>LIVBP</td>
<td>leucine/isoleucine/valine binding protein</td>
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<td>LTD</td>
<td>long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>nNOS</td>
<td>neuronal NO synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>n.s.</td>
<td>not significant</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>---------------------------------------------------------------------------</td>
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<tr>
<td>NRxy</td>
<td>NMDA receptor, x=subunit family,</td>
</tr>
<tr>
<td></td>
<td>y=splice variant or family member</td>
</tr>
<tr>
<td>OA</td>
<td>okadaic acid</td>
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<tr>
<td>PEAQX</td>
<td>(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-{(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077)</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP</td>
<td>protein phosphatase</td>
</tr>
<tr>
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<td>paired-pulse depression</td>
</tr>
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<td>PPF</td>
<td>paired-pulse facilitation</td>
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<tr>
<td>PSD</td>
<td>postsynaptic density</td>
</tr>
<tr>
<td>QA</td>
<td>quinolinic acid</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanolol.</td>
</tr>
<tr>
<td>SAP</td>
<td>synapse-associated protein</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>STA</td>
<td>staurosporine</td>
</tr>
</tbody>
</table>
Declaration

I declare that I myself carried out all the work in this thesis except where referenced and that it has not been submitted for any previous higher degree.
Publications

Papers


Abstracts


Chapter 1. Introduction

NMDA receptor: an historical perspective

The excitatory amino acid transmitter field has its historical origins in the finding of the convulsive effects of L-glutamate and L-aspartate upon mammalian brain tissue (Hayashi, 1952; 1954) and their depolarising and excitatory effects on central neurones (Curtis, 1959; 1960). However the concept that such an amino acid, found in relatively high concentrations in the central nervous system (CNS), may be responsible for the majority of neurotransmission only became accepted with the accumulation of later evidence from electrophysiological, pharmacological and molecular biological studies. L-glutamate is now considered the principal excitatory neurotransmitter in the mammalian CNS acting upon a variety of receptor types. The existence of glutamate receptor subtypes was suggested by the observations that glutamate agonist analogues showed different potencies on different subsets of neurones (Curtis & Johnston, 1974; McLennan, 1983). Furthermore, the development of several antagonists in the early 1970’s that had differential effects upon agonist responses resulted in an initial classification of NMDA, quisqualate and kainate receptors (Evans et al., 1978; Davies & Watkins, 1979; McLennan & Lodge, 1979; Ault et al., 1980). Since quisqualate was later shown to activate metabotropic glutamate receptors (Sladeczek et al., 1985; Nicoletti et al., 1986) and a
new agonist, \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid\ (AMPA) (Krogsgaard Larsen et al., 1980), was demonstrated to be selective for the quisqualate-activated non-NMDA receptors the ionotropic receptor family was reclassified as NMDA, AMPA and kainate (Collingridge & Lester, 1989). This has since been confirmed with the cloning and functional expression of various NMDA, AMPA and kainate receptor subunits (Hollmann & Heinemann, 1994).

**NMDA receptor physiology**

The NMDA receptor is a heteromeric ligand-gated ion channel that interacts with multiple intracellular proteins by way of different subunits (McBain & Mayer, 1994; Husi et al., 2000). NMDA receptors are involved in a variety of neural processes, including long-term potentiation (LTP) (Collingridge & Singer, 1990; Bliss & Collingridge, 1993), long-term depression (LTD) (Dudek & Bear, 1992; Mulkey & Malenka, 1992), learning, memory (Olney, 1990; Nakanishi et al., 1992), brain development, excitotoxicity (Choi & Rothman, 1990), neuropathologies such as epilepsy, psychosis and neurodegenerative diseases (Choi, 1988; Greenamyre & Young, 1989; Meldrum & Garthwaite, 1990; Doble, 1995). The NMDA receptor is characterised by a number of unique and important properties that distinguishes it from other ligand gated ion-channels. Firstly, extracellular \( \text{Mg}^{2+} \) at resting membrane potentials blocks the
NMDA receptor channel and it is only opened with simultaneous depolarisation and agonist binding (Mayer et al., 1984). Secondly, NMDA receptor channels are highly permeable to both monovalent cations and Ca\(^{2+}\) ions. NMDA receptor activation results in an influx of Ca\(^{2+}\) as well as the influx of Na\(^{+}\) and efflux of K\(^{+}\) that is characteristic of all excitatory amino acid (EAA) ionotropic receptors (MacDermott et al., 1986; Mayer & Westbrook, 1987; Schneggenburger et al., 1993). This characteristic Ca\(^{2+}\) entry is the key trigger for many important physiological phenomenon including LTP (Lynch et al., 1983; Bliss & Collingridge, 1993) and LTD (Dudek & Bear, 1992; Selig et al., 1995) where the relative magnitude of the rise in intracellular Ca\(^{2+}\) concentration and its temporal and spatial character determines which plasticity is induced (Lisman, 1989; Artola & Singer, 1993; Neveu & Zucker, 1996). Furthermore, prolonged activation of NMDA receptors results in excessive Ca\(^{2+}\) influx producing neuronal cell death in hypoxia, ischaemia and neurodegenerative disorders (Choi, 1988). Thirdly, the coincident binding of glutamate and the co-agonist glycine is necessary for receptor activation (e.g. two molecules of glycine and two molecules of glutamate in a tetrameric, heterodimeric, NR1/NR1/NR2/NR2 receptor) (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988). Glycine is present in cerebrospinal fluid at a concentration of about 10\(\mu\)M (Curtis & Johnston, 1974) and could be expected to attain a similar concentration in the extracellular fluid. At these concentrations the NMDA receptor could be fully saturated with
glycine, however it is conceivable that glycine is also released from glia or from the presynaptic terminal as a co-transmitter (Davanger et al., 1994). Furthermore, the widespread expression of mRNA for glycine transporter-1 throughout the brain, with a distribution colocalized to that of the NR1 subunit suggests that the concentration of glycine is closely regulated (Smith et al., 1992). It has been reported by some groups that the application of glycine potentiates NMDA receptor-mediated synaptic transmission (Danysz et al., 1989; Wilcox et al., 1996; Lim et al., 2004) but not by others (Fletcher & Lodge, 1988; Obrenovitch et al., 1997).

**NMDA receptor molecular biology**

Molecular studies have reported that native rat NMDA receptors may be either tetrameric or pentameric membrane proteins comprising the NMDA receptor 1 (NR1) subunit family, of which there are eight splice variants (NR1a-h based upon the presence or absence of 3 exons) encoded by a single gene, and the NR2 subunit family, of which there are four members (NR2A-D) (Laube et al., 1998), each encoded by four distinct genes (Seeburg et al., 1994; Dingledine et al., 1999). A third family of NMDA receptor subunits, NR3, has also been described (Ciabarra et al., 1995; Sucher et al., 1995); of which there are two types NR3A and NR3B that less commonly contribute to NMDA receptors (Nishi et al., 2001; Chatterton et al., 2002; Eriksson et al., 2002). The NR3A subunit has been
shown to function during early development and to modulate negatively ion channel activity (Das et al., 1998). It has been reported that mutations in the NR2 subunit alters the NMDA receptor’s response to glutamate whilst mutations in the NR1 subunit do not (Laube et al., 1997; Anson et al., 1998; Anson et al., 2000). This is consistent with the pronounced glutamatergic heterogeneity of the native NMDA receptor being dependant upon the NR2A-D subunit complement and the slight heterogeneity seen with NR1 splice variants (Buller & Monaghan, 1997). In particular, the presence of different NR2A-D subunits within a heteromeric receptor confers different degrees of affinity for glutamate and dictates the functional properties of the receptor (table 2). Thus, the NR2 subunit contains the glutamate binding site (Laube et al., 1997) whilst the co-agonist glycine’s binding site is located on the NR1 and the NR3 subunit (Kuryatov et al., 1994). Native heteromeric receptor complexes are thought to be combinations of one or two NR1 and/or NR3 subunits forming the glycine-binding site and two or three NR2 subunits providing the glutamate-binding site. The exact subunit composition influencing the NMDA receptors’ pharmacology and function (Kohr & Seeburg, 1996; Krupp et al., 1996; Monaghan & Larsen, 1997; Vicini et al., 1998; Pizzi et al., 1999). The subunit composition of the NMDA receptor is dynamic, changing during synaptic development (Kew et al., 1998a; Tovar & Westbrook, 1999), synaptic plasticity (Kiyama et al., 1998; Manabe et al., 2000) and other physiological and pathophysiological processes. The
activity-dependent trafficking of NMDA receptors is achieved both by receptors recycling through exocytosis-endocytosis from intracellular sites and lateral diffusion of receptors between synaptic and extrasynaptic sites (Tovar & Westbrook, 1999; Grosshans et al., 2002; Groc et al., 2004; Lavezzari et al., 2004).

**NMDA receptor distribution**

Glutamate receptors are predominantly expressed in the CNS, but have also been reported in pancreatic islet cells, osteoclasts, osteoblasts, nerve terminals in the skin, mast cells, taste buds, cardiac ganglia and even in plant cells. The first evidence for differential NMDA receptor distribution came from electrophysiological studies into the endogenous agonist quinolinic acid (QA), which demonstrated variable potency in different brain regions (Stone & Burton, 1988). In the brain NMDA receptors are now known to be heterogeneously distributed according to their subunit composition, in particular to that of the NR2 family (Monaghan & Buller, 1994) (see table 1). The NR1 subunit is expressed throughout the brain with little differential distribution of splice variants (Nakanishi et al., 1992; Laurie & Seeburg, 1994) The NR2A-D subunits are expressed in a very distinct pattern (Watanabe et al., 1993; Buller et al., 1994; Laurie & Seeburg, 1994). In the adult brain the NR1 and NR2A subunits are ubiquitous, the NR2B subunit is expressed mainly in the forebrain, NR2C
is found particularly in the cerebellum and various select nuclei and NR2D expression is limited to the diencephalon and the mid-brain (McBain & Mayer, 1994). Hippocampal CA1 pyramidal cells express mRNA for NR1, NR2A, NR2B and NR2D in the adult human (Scherzer et al., 1997) and the juvenile rat (Kirson et al., 1999).

NMDA receptors have been amply demonstrated on the postsynaptic membrane (Petralia et al., 1994; Racca et al., 2000), and there is accumulating evidence suggesting a presynaptic locus. AMPA, mGlu and kainate receptors have all been established as presynaptic autoreceptors in the hippocampus (Vazquez et al., 1994; Chittajallu et al., 1996; Alici et al., 1997), modulating excitatory transmission by altering glutamate release probability. Immunohistochemical studies have suggested the existence of presynaptic NMDA autoreceptors (Liu et al., 1994; Paquet & Smith, 2000). Also, functional studies have reported evidence for presynaptic NMDA receptors in the cerebellum (Glitsch & Marty, 1999), visual cortex (Sjostrom et al., 2003), spinal cord (Liu et al., 1997), hippocampus (Breukel et al., 1998), suprachiasmatic nucleus (Hamada et al., 1998) and entorhinal cortex (Berretta & Jones, 1996). Presynaptic NMDA receptors have been reported to be NR2B subunit-containing and tonically activated, causing an increase in glutamate release probability, dependent upon Ca$^{2+}$ influx, in the entorhinal cortex (Woodhall et al., 2001).
**NMDA receptor development**

Developmentally only NR2B and NR2D subunits are present and widespread in the prenatal brain but shortly after birth, NR2A and NR2C subunits quickly predominate while the NR2B and NR2D subunits decline to adult levels and a limited distribution. NR2B subunits are expressed from late embryonic stages up to adulthood, while expression of NR2A subunits only develops during early postnatal stages and rises to mature levels by post-natal day 21 (Williams et al., 1993; Mori & Mishina, 1995; Wenzel et al., 1995; Portera Cailliau et al., 1996). This transformation is particularly significant for the age-dependence of synaptic plasticity (Kemp et al., 2000) and glutamate toxicity (Liu et al., 1996). NR1 subunits are present and widespread both pre- and post-natally (Lynch & Guttmann, 2001)(see tables 1, 2 and 3).
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Localisation in adult animals</th>
<th>Developmental course</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1</td>
<td>Ubiquitous with splice variant distribution limited to specific cells</td>
<td>Prenatal and postnatal</td>
</tr>
<tr>
<td>NR2A</td>
<td>Widespread throughout the forebrain. Less prominent in the basal ganglia</td>
<td>Develops post-natally</td>
</tr>
<tr>
<td>NR2B</td>
<td>Largely limited to forebrain</td>
<td>Widespread with later decline to adult levels and distribution</td>
</tr>
<tr>
<td>NR2C</td>
<td>Largely limited to cerebellum</td>
<td>Appears postnatally</td>
</tr>
<tr>
<td>NR2D</td>
<td>Isolated cells of thalamus, sub thalamus and nNOS containing cells of cortex</td>
<td>Widespread prenatally</td>
</tr>
</tbody>
</table>

Table 1. Summary of the distribution and developmental course of NMDA receptor subunits.
<table>
<thead>
<tr>
<th>NMDA receptor property</th>
<th>Subunit heterogeneity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate and glycine affinity</td>
<td>2D &gt; 2B &gt; 2C</td>
</tr>
<tr>
<td>Channel open probability</td>
<td>2A (2-5 fold higher) &gt; 2B</td>
</tr>
<tr>
<td>Peak current density</td>
<td>2A (4 times larger) &gt; 2B</td>
</tr>
<tr>
<td>Channel conductance</td>
<td>2A, 2B &gt;&gt; 2C, 2D</td>
</tr>
<tr>
<td>Receptor kinetics</td>
<td>2A &gt; 2B = 2C &gt; 2D</td>
</tr>
<tr>
<td>Current decay time</td>
<td>2A &gt; 2B &gt; 2C &gt; 2D</td>
</tr>
<tr>
<td>Ca(^{2+}) influx</td>
<td>2B &gt; 2A</td>
</tr>
<tr>
<td>Sensitivity to Mg(^{2+}) block</td>
<td>2A, 2B &gt;&gt; 2C, 2D</td>
</tr>
<tr>
<td>Deactivation time</td>
<td>2D &gt;&gt; 2B, 2C &gt; 2A</td>
</tr>
<tr>
<td>Desensitisation sensitivity</td>
<td>2A &gt;&gt; 2D &gt;&gt; 2B, 2C</td>
</tr>
<tr>
<td>Speed of recovery from desensitisation</td>
<td>2A &gt; 2A/2B &gt; 2B</td>
</tr>
<tr>
<td>Proton sensitivity</td>
<td>2A &gt; 2B &gt; 2D &gt; 2C</td>
</tr>
</tbody>
</table>

Table 2. Summary of the functional heterogeneity of NMDA receptors depending on the presence of specific NR2 subunits.
| **NMDA** | Widely distributed in mammalian CNS (especially enriched in hippocampus and cerebral cortex). |
| **AMPA** | Widespread in CNS; similar distribution to NMDA receptors. |
| **Kainate** | Concentrated in a few specific areas of CNS, corresponding to NMDA and AMPA receptor distribution. |
| **Metabotropic** | A class of receptors positively linked to inositol triphosphate or negatively to cyclic AMP formation. |

Table 3. Summary of the general distribution of excitatory amino acid (EEA) receptors in the mammalian CNS.
NMDA receptor pharmacology

The complicated physiology of the NMDA receptor complex provides agonists and antagonists with several distinct binding sites: glutamate, strychnine-insensitive glycine, ion-channel, polyamine and other modulatory sites (Figure 1).

Glutamate, NMDA, aspartate, quinolinic acid, homoquinolinic acid and other agonists activate the glutamate site; the prototypic antagonist of this site is 2-AP5. The glycine site is activated by glycine (Johnson & Ascher, 1987) and several antagonists exist including ACBC, CGP 78608 and 7-chlorokynurenic acid (Fletcher & Lodge, 1988; Kemp et al., 1988). The NMDA receptor ion channel is blocked by Mg\(^{2+}\) ions at resting membrane potentials. Other modulators have also been discovered that block the ion channel in a non-competitive manner; notably, dizocilpine (MK 801), PCP and ketamine. Zinc, like other group IIB metal ions produces inhibition of the NMDA receptor and is selective at nanomolar concentrations to NR2A subunit-containing NMDA receptors (Paoletti et al., 1997). Polyamines such as spermine and spermidine can inhibit or potentiate the NMDA receptor, at high and low concentrations respectively. Polyamines promote channel opening at low micromolar concentrations by glycine-dependent and glycine-independent mechanisms (Rock & Macdonald, 1992; Williams, 1997). At higher concentrations, they block the channel in a
voltage-dependent manner. Antagonists at this site, inhibiting the NMDA receptor, include ifenprodil and haloperidol (Reynolds & Miller, 1989; Ilyin et al., 1996; Lynch & Gallagher, 1996). Modulation also occurs at the Ro 25-6981 site, the redox site and with ethanol and the volatile anaesthetics.
Figure 1. The NMDA receptor.

A model showing the agonist binding sites for glutamate and glycine and the competitive antagonists that can block these sites. Additionally, other modulators can block the ion-channel and modulate the receptor at discrete sites (Kemp & McKernan, 2002).
AMPA and kainate receptors

The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (KA) receptors are the non-NMDA ionotropic receptor subtypes that activate Na⁺ and K⁺ conductances whilst the NMDA receptors activate Na⁺, K⁺ and Ca⁺ conductances (Monaghan et al., 1989; Barnard & Henley, 1990). AMPA receptors are hetero-oligomeric proteins consisting of the subunits GluR1 to GluR4, each receptor comprising four subunits (Hollmann & Heinemann, 1994; Rosenmund et al., 1998). The AMPA and kainate receptors have at least three separate binding sites at which agonists and antagonists can act: glutamate binding, desensitisation and intra-ion channel binding sites. Kainate receptors consist of GluR5-7 and KA1-2 subunits, and have approximately 40% sequence homology to AMPA receptor GluR1-4 subunits. High affinity binding of kainate is observed to the KA1 and KA2 subunits compared to low affinity binding to GluR5-7. The same agonists and antagonists can modulate both AMPA and kainate receptors with little differentiation. However, certain desensitisation inhibitors, which act allosterically to modulate positively sites on the ion channel, can distinguish between AMPA and kainate receptors (Table 3).
Inhibitory GABA-ergic transmission

As well as excitatory transmission mediated by glutamate there is also the presence of GABA-ergic interneurones that mediate feedforward and feedback inhibition of excitatory transmission. Activation of these inhibitory interneurones by glutamate results in a dual component inhibitory postsynaptic potential (IPSP)(Alger & Nicoll, 1982; Benardo, 1994). This consists of a GABA_A-mediated fast component which results in the activation of GABA_A ionotropic receptors releasing hyperpolarising Cl⁻ ions into the neurone. The other component is a slow GABA_B-mediated component which results in the activation of the GABA_B metabotropic receptor (Alger, 1984) leading to the hyperpolarising influx of K⁺ ions and the negative modulation of the influx of Ca²⁺ ions. IPSPs can inhibit excitatory synaptic transmission by moving the membrane potential to values that are more negative and increasing the amount of depolarisation required by EPSPs to reach the threshold for action potential generation. GABA_B mediated slow IPSPs are possibly better poised to counter longer-lasting EPSP_NMDA which because of their long slow kinetics have been reported to escape fast GABA_A inhibition (Benardo, 1995) although one report suggested that when stimulation evokes only a small EPSP (2-5mV) the GABA_B component is negligible (Otmakhova & Lisman, 2004). In addition to postsynaptic GABA receptors there is also a presynaptic GABA_B autoreceptor in the Schaffer collateral feedforward...
interneurones (but not in the perforant pathway). When the interneurone is repeatedly activated, GABA is released in sufficient quantity to feedback on these GABA$_B$ receptors, which inhibits the release of further GABA, and synaptic inhibition is depressed (Davies et al., 1990).
Excitotoxicity

The term excitotoxicity was first coined by John Olney who observed that a number of excitatory amino acids (EAAs) killed neurones in the hypothalamus with a potency that correlated with their efficacy in depolarising cells. Excitotoxicity is a critical mechanism contributing to neurodegeneration during ischaemia and hypoxia. Both the loss of calcium homeostasis and the activation of EAA receptors are postulated to be responsible for excitotoxic neuronal damage (Olney, 1969; 1986; Seisjo, 1988; Choi, 1991; Sattler et al., 1999). With the activation of NMDA receptors both of these hypotheses are fulfilled. The over-activation of NMDA receptors leads to an influx of Ca$^{2+}$ resulting in necrosis or apoptosis by several proposed mechanisms. The subsequent activation of Ca$^{2+}$-sensitive enzymes (nNOS, endonucleases and proteases), the production of highly reactive free radical species, mitochondrial impairment and the activation of immediate-early response sense genes all contributing to neuronal death. Blockade of this influx, by NMDA antagonists and calcium antagonists, is neuroprotective in animal models of stroke and seizure (Lee et al., 1999). The clinical use of non-selective NMDA receptors as neuroprotective drugs has been hindered by their narrow therapeutic window, which produces adverse drug reactions including sedation, muscular weakness, ataxia, confusion and tinnitus. Subunit selective antagonists such as ifenprodil have been associated with a
decrease in side effects allowing an effective therapeutic dose to be titrated (Gotti et al., 1988). Nevertheless, therapeutic NMDA receptor antagonists have yet to be developed with clinical success (De Keyser et al., 1999). Several studies have specifically implicated both the NR2A- and NR2B-containing NMDA receptors in excitotoxicity. Excitotoxicity in cultured embryonic forebrain neurones coincides with a dramatic increase in NR2B mRNA levels during the first 10 days, thereafter remaining stable (Cheng et al., 1999). However, decreased expression of NR2A and NR2B mRNA has also been reported in CA1 and other hippocampal areas, in response to a severe ischaemic insult, altering the function of the NMDA receptors (Zhang et al., 1997). In addition to changes in subunit expression, ischaemia has also been shown to increase the tyrosine phosphorylation of both the NR2A and NR2B subunits thereby altering their function (Kim et al., 1998; Cheung et al., 2000).
Anatomy of the hippocampus

The electrophysiological experiments described in this study were carried out in the hippocampal slice preparation of the 7-8 week old Wistar rat. The hippocampus along with the amygdala, adjacent regions of the limbic cortex and the septal area constitute the main structures of the limbic system. These structures form major interconnections with portions of the thalamus and the cerebral cortex (cingulated gyrus).

For three reasons the hippocampus is one of the most studied areas of central nervous system. Firstly, it has an easily identifiable structure at the gross and histological levels. Secondly, it has been recognized that the hippocampus plays a fundamental role in learning and memory (patients who have had bilateral hippocampaelctomy have suffered from a permanent loss of storing new information into their long-term memory). Thirdly, the hippocampus is especially susceptible to seizure disorders and is particularly vulnerable to the effects of ischaemia and hypoxia (Johnston & Amaral, 1998).

In the brains of mammals, the hippocampus is a bilateral structure that appears as a ridge extending into the lateral ventricle. The outer surface of the hippocampus is composed of myelinated fibres, appears white and is called the alveus. The hippocampal formation can be divided into Ammon’s horn, the subiculum, the dentate gyrus and the entorhinal cortex.
Based on the Golgi method of staining, the anatomist Lorente de Nó (1934) (see Johnstone & Amaral, 1998) divided Ammon's horn into four cornu Ammonis subfields: CA1 to CA4. The designation CA4 is no longer used because it referred to the region occupied by the polymorphic layer of the dentate gyrus. CA1 is equivalent to the regio superior and CA2 and CA3 fields are equivalent to the regio inferior. A narrow transitional zone, CA2, separates CA1 and CA3 (Figure 2).

The dentate gyrus contains round, tightly packed neurons called granule cells and consists of three layers: the granule layer, which is the principal layer, the molecular layer above the granule cell layer and a polymorphic layer below the granule cell layer (Isaccson, 1987).

In all CA fields below the alveus is the stratum oriens, which contains the basal dendrites of the pyramidal cells. The cell bodies are clearly visible under a microscope as a dark band. Below this is the stratum radiatum consisting of the apical dendrites of the pyramidal cells and the Schaffer collaterals, which are collateral branches from axons of pyramidal cells in the CA3 region (Cajal, 1968).
Neuronal circuits in the hippocampus

The excitatory neuronal circuitry of the hippocampal formation is illustrated in figure 2. The functional organisation of the hippocampus has been described in terms of a ‘trisynaptic’ circuit. Information that flows from the neocortex into and out of the hippocampus travels in a unidirectional manner through this trisynaptic pathway. The entorhinal cortex is considered the starting point of the circuit. Neurones located in layer II of the entorhinal cortex give rise to the perforant pathway that projects through the subiculum and terminates both in the dentate gyrus and in the CA3 region. Neurones forming the medial entorhinal cortex produce axons that terminate in the middle layer of the molecular layer of the dentate gyrus; the outer third of the molecular layer receive axons from the lateral entorhinal cortex. These two components of the perforant pathway also terminate in the stratum lacunosum-molculare of the CA2 and CA3 regions. Neurones located in layer III of the entorhinal cortex project to the CA1 and the subiculum.

The granule cells also synapse onto neurones of the polymorphic layer, which supplies associational connections to other levels of the dentate gyrus. The CA3 pyramidal cells project heavily within the CA3 and as the Schaffer collateral projection to the CA1 stratum radiatum and stratum oriens. CA1 neurones project to both the subiculum and the deep layers of
the entorhinal cortex, which in turn produce projections back into many of the cortical areas that originally terminated in the entorhinal cortex. Therefore information sent from cortical neurones to the entorhinal cortex can then negotiate the complete hippocampal circuit and be returned back to its cortical origin (Witter, 1989).

As well as the main excitatory circuitry, there are also inhibitory pathways throughout the hippocampus. Three types of interneurones control this circuit in the pyramidal cell layers: axo-axonic cells, basket cells and bistratified cells. The majority of interneurones in the hippocampus use the inhibitory transmitter γ-aminobutyric acid (GABA). The axo-axonic cells synapse upon the initial segment of pyramidal neurones and have a strong influence over the generation of action potentials. Basket cells synapse onto the somata and bistratified cell synapse at apical and basal dendrites of the pyramidal neurones. The dendrites of these three classes of interneurone project into the stratum radiatum and stratum oriens where they can encounter excitatory inputs from the Schaffer collaterals, commissural-association fibres and feedback synapses from local pyramidal neurones (Isaccson, 1987; Johnston, 1998).
Figure 2. Diagram showing the anatomy of the rat hippocampal formation and the placement of the stimulating and recording electrodes.

A, location of the left hippocampus in rat brain. B, structure of a hippocampal slice showing the principal excitatory neurons. The stimulating electrode (1) was positioned in the stratum radiatum at the CA1/CA2 junction. Orthodromic population spikes and excitatory postsynaptic potentials were recorded from the stratum pyramidale and stratum radiatum respectively using electrodes (2) and (3) placed in the CA1 region. C, hippocampal circuitry. DG, dentate gyrus; ENT, entorhinal cortex; mf, mossy fibre; pp, perforant pathway; rc, recurrent collateral; sc, Schaffer collateral; SUB, subiculum. (Revest & Longstaff, 1998).
The hippocampal slice preparation

Ever since cortical slices were demonstrated to have comparable electrical activity with the intact animal (Yamamoto & McIlwain, 1966) the slice preparation has been used extensively in the study of synaptic transmission and electrophysiological experiments in the mammalian CNS. The studies leading to our present understanding of how NMDA, AMPA, and metabotropic glutamate receptors contribute to synaptic transmission; and investigations of LTD and LTP have come almost exclusively from brain slice studies. Hippocampal slices have also shown good conditions to allow intracellular recording (Yamamoto, 1972; Kerkut, 1981). The hippocampus is an essentially laminar structure, each transverse slice having the same ‘trisynaptic’ circuitry. The neuronal function and anatomy of the slice preparation, in the plane in which it is cut, is calculated to be similar to the intact brain producing representative electrophysiological and pharmacological responses. The slices should be well enough prepared to allow recording at physiological temperatures (36-37°C for rodent slices) however, this is not usually achievable and a sub-physiological temperature of 28-30°C is normally used.
Advantages of the in vitro slice preparation over in vivo techniques include:

1. Technical simplicity: it is relatively easy to record via extracellular, intracellular and whole-cell patch-clamp compared with in vivo experiments.

2. Control over the conditions of the preparation. If one can reduce the number of contributory variables, greater significance can be drawn upon results from a constant environment (e.g. temperature, movement, $O_2/CO_2$ saturation, absence of the influence of an anaesthetic maintenance dose).

3. Improved visualization of tissue. The tissue is easily identified under low magnification facilitating the positioning of stimulating and recording electrodes.

4. Control of the extracellular environment and the field potential to study neurophysiology and pharmacology. This is helpful when applying drugs and doses that are impractical in whole animal studies.

5. Humane treatment of animals, because slice preparation does not require restraint or the maintenance of physiological functions it is a relatively more humane method.
6. The ability to use technically demanding methods that are difficult or impossible in intact preparations. For example, the ability to isolate specific pathways and record from specific postsynaptic cells without contamination of other synaptic inputs from other brain regions which have been dissected (Grover & Teyler, 1990), or the ability to use optical techniques.

These advantages of the slice preparation have led to increasing numbers of neuroscientists to use this technique. However, there are also some disadvantages with brain slice preparations:

1. The dissection and slicing process severs the hippocampal slices’ normal sensory input and motor output. Long feedback loops, some excitatory pathways and some inhibitory pathways are also lost. One obvious symptom of this is the markedly lower levels of spontaneous activity found in the slice preparation compared to the intact animal. This brings into question the similarity and relevance of the in vitro slice preparation compared to the in vivo environment. Furthermore, it has been demonstrated that phenomena such as LTD induction are more readily induced in vitro than in vivo (Bear & Abraham, 1996)
2. The crude method of slicing causes damage along the faces of the slice, this results in cell damage and the release of substances that may affect the slice.

3. The tissue is subject to an anoxic period during preparation (Lipton & Whittingham, 1979).

4. The ionic environment does not mimic exactly the normal extracellular conditions in vivo. This is of particular relevance in the induction and maintenance of LTD and LTP that are dependent upon relative \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) ion concentrations. The continuous perfusion of artificial cerebrospinal fluid (ACSF) can also wash out any modulatory compounds endogenous to the slice (such as growth factors released following stimulation).

5. The addition of drugs to the artificial cerebrospinal fluid (ACSF) results in the exposure of the whole slice to pharmacological modulation. This confounds the significance of results seen with the subset of cells that are being recorded.

6. Finally, the diversity of methods used in each brain slice laboratory makes the comparison of findings difficult (Lipton et al., 1995). The most obvious differences include: (A) the maintenance of brain slices at the interface of the ACSF and oxygenated atmosphere.
versus those maintained entirely submerged in ACSF, (B) cutting brain slices on a manual tissue chopper versus a vibratome or a vibraslice, (C) differing ACSF recipes and (D) temperature differences.

**Extracellular responses**

Single electrical stimulations of the Schaffer collaterals result in a distinctive sequence of excitation followed by inhibition in the target CA1 pyramidal neurones. Field extracellular recordings represent the summed responses from a population of neurones close to the recording electrode. The field excitatory postsynaptic potential (fEPSP) recorded in the stratum radiatum consists of an initial fibre volley of action potentials in the presynaptic fibres producing a negative-going transient; this is followed by a slower negative-going potential with a time-period similar to that of the underlying synaptic currents. The current flowing into these dendrites during the fEPSP will exit the neurons near the cell body layer in the stratum pyramidale where a recording electrode will record a concurrent positive going potential. If the strength of the synaptic input is adequate to evoke action potentials in the neurons, then a negative-going potential (population spike) can also be recorded in the stratum pyramidale resulting from the inward current during the postsynaptic action potentials.
Paired-pulse interactions

The magnitude of synaptic transmission in the mammalian brain can be modulated in different ways. Two examples of such modulation are paired-pulse facilitation (PPF) and paired-pulse depression (PPD).

Paired-pulse facilitation

Paired-pulse facilitation (PPF) is a form of short-term plasticity in which the synaptic response to the test pulse (second stimulus) given after the conditioning pulse (first stimulus) is enhanced in comparison to the conditioning pulse when interstimulus intervals are relatively long (≥20ms). The most widely accepted hypothesis to explain the mechanism of PPF is based on the accumulation of presynaptic Ca\(^{2+}\) after the first stimulation. The first pulse induces a Ca\(^{2+}\) influx that lingers in the presynaptic terminal for several hundred milliseconds. This residual Ca\(^{2+}\) combined with fresh Ca\(^{2+}\) entering during the second pulse leads to the increased transmitter release (Katz & Miledi, 1968; Thomson, 2000; Zucker & Regehr, 2002). The Ca\(^{2+}\) concentration in the presynaptic nerve terminal affects directly the release probability of neurotransmitter. Furthermore, reports at the neuromuscular junction have suggested that the second of the two action potentials induces release with higher probability than the first one because of a small but persistent increase of the intracellular Ca\(^{2+}\) concentration in the axon terminal. This residual Ca\(^{2+}\)
can enhance the release probability by increasing the fusion of synaptic vesicles with the presynaptic membrane and enhancing the number of vesicles released by the action potential (Katz & Miledi, 1968; Mennerick & Zorumski, 1995; Debanne et al., 1996). Neuromodulators and physiological processes that alter transmitter release probability also affect the paired-pulse ratio. Increasing the external Mg\textsuperscript{2+}/Ca\textsuperscript{2+} ratio (Davies & Collingridge, 1993; Lambert & Wilson, 1994; Wilcox & Dichter, 1994) and applying adenosine (Lupica et al., 1992; Higgins & Stone, 1995) decreases the probability of release of neurotransmitter during the first stimulus and increases the probability of release by the second stimulus producing enhanced PPF. In the hippocampus, PPF of excitatory synaptic potentials in the CA1 and CA3 is observed when large numbers of axon are concurrently stimulated (Creager et al., 1980; Manabe et al., 1993) whereas, during conditions in which the release probability is enhanced by decreasing the Mg\textsuperscript{2+}/Ca\textsuperscript{2+} ratio then PPF is reduced (Nathan et al., 1990; Kahle & Cotman, 1993).

**Paired-pulse depression**

When two evoked potentials are elicited when interstimulus intervals are small (≤10ms), the size of the second response is smaller than the first, a phenomenon called paired-pulse depression (PPD). The mechanism underlying PPD of synaptic transmission is unclear. One hypothesis is the activation of GABA\textsubscript{B} autoreceptors (Deisz & Prince, 1989), but studies
have shown that GABA_B receptor antagonists did not attenuate PPD of unitary inhibitory post synaptic potentials (IPSPs) in pairs of cultured hippocampal neurons (Wilcox & Dichter, 1994). An alternative presynaptic mechanism for PPD is a transient decrease in the quantal content caused by depletion of the readily releasable vesicle pool by the first stimulus (Mennerick & Zorumski, 1995; Stevens & Wang, 1995; Debanne et al., 1996). Postsynaptic mechanisms such as the desensitisation of GABA_A receptors (Alger, 1991) and a reduced driving force due to intracellular accumulation of chlorine ions and/or extracellular accumulation of potassium ions may also explain PPD (McCarren & Alger, 1985). Since paired-pulse depression is observed most clearly and reliably when the interstimulus interval is around 15–100 ms, which corresponds to the time course of the GABA_A mediated fast inhibitory potential recorded intracellularly in pyramidal cells (Davies et al., 1990), it is thought to be largely caused by the activation of GABA_A receptors (Wilcox & Dichter, 1994). It is difficult to test this proposition using bath application of GABA_A receptor antagonists such as bicuculline which increase the excitability of the pyramidal cells by blocking the effects of spontaneously released GABA.
Whilst changes in paired-pulse interactions are widely considered to be due to presynaptic alterations in release probability; when studied on fEPSPs and especially where there are depolarisation-induced changes in the size of the postsynaptic potential in response to the test agents many factors affect them which can confound the interpretation. These include changes in presynaptic release probability (Andreasen & Hablitz, 1994), activity of presynaptic autoreceptors on inhibitory or excitatory terminals (Davies et al., 1990; Stanford et al., 1995), modulation of postsynaptic conductances (e.g. voltage-dependent NMDA receptor) and the presence of GABA-ergic inhibition following the fEPSP and the depression cause by NMDA agonist action (Duguid & Smart, 2004). In particular, it has been reported that there is a non-linear inverse relationship between feedforward inhibition and the NMDA dependent PPF component of the fEPSP (Papatheodoropoulous & Kostopoulos, 1998) although, this NMDA-component of PPF is manifested by an increase in the duration of the fEPSP and not the amplitude of the slope.

In hippocampal slices paired-pulse inhibition has been reported at interstimulus intervals less than 40 ms and in the present experiments at less than 20 ms while PPF is observed at longer time intervals (20 and 50 ms) (Lynch et al., 1983; Higgins & Stone, 1995; Nikbakht & Stone, 2000).
Aims

1. To investigate NMDA receptor subunit pharmacology in the intact, adult hippocampal slice preparation using the newly developed NR2A and NR2B subunit-selective NMDA receptor antagonists Ro 25-6981 and PEAQX.

2. To address the contribution of NR2A and NR2B subunit-containing NMDA receptors in the induction of NMDA receptor dependent synaptic plasticity in the Schaffer collateral-CA1 pyramidal synapse.

3. A report had suggested that the NR2A/2B subunit selective NMDA receptor agonist homoquinolinic acid was binding to a novel NMDA-insensitive site in the brain. An investigation of NMDA insensitive effects on the fEPSP was undertaken to reveal any residual effects. Furthermore, the selectivity of homoquinolinic acid allowed the investigation of the NR2A and NR2B components of the previous study (1) and to find evidence for the involvement of the NR2D subunit. In addition, this study investigated changes of paired-pulse interactions using these compounds.
Chapter 2. Material and methods

Preparation of hippocampal slices

Male Wistar rats (130-180g, 7-8 weeks) were anaesthetised by an intraperitoneal injection of urethane solution (1.5 g/Kg); urethane has been shown not to interfere with synaptic plasticity, a problem encountered when using phenobarbitone (unpublished observations). The animals were then killed by cervical dislocation, decapitated using a guillotine, and the brain gently and rapidly removed to ice-cold and oxygenated artificial cerebrospinal fluid (ACSF). The intact brain was kept cold and moist by copious amounts of ACSF whilst it was transferred to a petri dish lined with filter paper to prevent sliding. The cerebellum was removed and the two cerebral hemispheres were separated with a scalpel blade. Each hippocampus was dissected free from the surrounding tissue using small spatulas and cut transversely using a McIlwain tissue chopper into slices 450μm thick, perpendicular to their longitudinal axis. The slices were gently separated from each other using blunt glass microelectrodes to ensure that they receive adequate amounts of oxygen and nutrients and to allow individual slices to be transferred easily. The slices were incubated at room temperature (21-23°C) on a fresh filter paper lined petri dish containing a small amount of freshly gassed ACSF to cover the slices. The petri dish was kept in an incubation chamber saturated in an atmosphere of
95% O₂ and 5% CO₂ for at least 1 hour prior to individual slices being transferred to the recording chamber.

**Composition of ACSF**

The composition of the ACSF was (in mM): KH₂PO₄ 2.2, KCl 2, NaHCO₃ 25, NaCl 115, CaCl₂ 2.5, MgSO₄ 1.2, and glucose 10. It was gassed with a mixture of 95% O₂ and 5% CO₂ yielding a pH ~7.4.

**Bath superfusion and application of drugs**

Following incubation, individual slices were transferred to a 1 ml submerged recording chamber using a wide tipped plastic Pasteur pipette. A seeker wire, the end of which was modified in such a way as to form a shape was used to gently hold the slice in place. The slice was left to stabilise whilst being continuously superfused with ACSF by a gravity-fed silicone tube at a rate of 4 ml/min. The ACSF was kept saturated with 95% O₂ and 5% CO₂ and heated using a thermostatically controlled water bath to 28-30°C. Drugs were added to the ACSF and fed via a three-way tap through the silicon tubing to the recording chamber. Due to the length of tubing, it took 1 minute for fluid to reach the recording chamber. To prevent the build-up of organic matter the silicon tubing and recording chamber were flushed with distilled water before and after use. Additionally, the system was more aggressively cleaned using diluted...
bleach once a month and the tubing replaced every six months and as required.

**Stimulation**

Stimuli were AC amplified square wave constant-current pluses of 300μs duration. Paired stimuli, where used, were delivered through the same electrode. The slices were stimulated using a concentric bipolar electrode (Clark Electromedical Instruments Ltd, Harvard Apparatus) positioned in the stratum radiatum near the commissural border of CA1/CA2 for orthodromic activation of pyramidal cells. The slice was briefly and occasionally stimulated at 1 Hertz (Hz) until a population of neurones was identified; once this was achieved, a stimulus frequency of 0.05Hz was used. The population of neurones was then allowed to stabilise. Population spikes were recorded at 70% of maximum amplitude whilst fEPSP slopes were recorded at 50% of their maximum amplitudes. Only field responses that produced clear fEPSP and population spikes with maximum amplitudes greater than 2mV were used in experiments. Furthermore, responses that showed greater than 5% drift over the 20-minute baseline-recording period were excluded (Figure 3).
Figure 3. Example responses recorded from the CA1 region of the rat hippocampal slice.

Trace (A) shows a population spike recorded from the stratum pyramidale and trace (B) shows a fEPSP, recorded from the stratum radiatum.
Recording

Extracellular population spike potentials and field excitatory postsynaptic potentials (fEPSPs) were recorded extracellularly from the stratum pyramidale and the stratum radiatum, respectively, using borosilicate glass microelectrodes, which were produced on a Kopf vertical puller. The electrode tips were broken back to produce an opening 2-4μm in diameter under low magnification using a glass probe, resistances approximately 2–5MΩ. The electrodes were filled with sodium chloride 0.9% solution using a fine 36-gauge needle.

Evoked responses were amplified through a Neurolog system, displayed on a digital oscilloscope and recorded onto a personal computer via a Cambridge Electronic Device (CED) micro 1401 interface and Signal analysis software (Cambridge Electronic Design, version 1). Responses were filtered between DC and 5kHz; line frequency interference was removed by digitally filtering (Humbug, Digitimer Ltd).
Data analysis

Responses were quantified as the amplitude of the population spike in mV (measured as the difference between peak negativity and the averaged values of the two peaks of the positive-going synaptic potential) and the fEPSP was measured as the maximum slope of the initial negative going gradient with two static cursors using Signal software; manually selecting and recording the slope in response to changes in the size of the fEPSP response and electrical interference (figure 3). Individual responses were measured, normalised and compared to the 20-minute initial baseline recordings. Every time point was pooled and graphed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA, version 3) showing mean ± standard error of the mean (s.e.m.). Rarely, single responses had to be excluded due to anomalous electrical interference disrupting the recording of evoked responses.

When paired-pulse interactions were investigated, inhibition or facilitation was expressed as the ratio of the second response of a pair compared with the first response.

Statistical analysis

The statistical significance of a difference was assessed on raw data by a paired Student's t-test or, if the difference was between three or more
means, significance was calculated with a one-way or repeated measures analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-test using GraphPad Prism software; p values less than 0.05 were considered statistically significant. IC\textsubscript{50} and EC\textsubscript{50} values were calculated using GraphPad Prism software by generating non-linear regression, sigmoidal concentration-response curves. In the figures statistically differences between data points are sometimes indicated by asterisks: * p<0.05; ** p<0.01; *** p<0.001.

**Chemical agents and drugs**

Constituents of ACSF were of anaal grade and were dissolved in distilled water to prepare fresh every day. N-Methyl-D-aspartic acid (NMDA), D-(-)-2-amino-5-phosphonopentanoic acid (2-AP5), dizocilpine (MK 801), zinc acetate (Zn\textsuperscript{2+}), (RS)-AMPA hydrobromide (AMPA) and kainate (KA) were dissolved in distilled water to obtain stock solutions. Quinolinic acid (QA) and homoquinolinic acid (HQA) were dissolved in 1 molar equivalencies of NaOH to give stock solutions. Dimethyl sulphoxide (DMSO) was used to dissolve kynurenic acid (KYA), 2-carboxy-3-carboxymethylquinoline (CCMQ), okadaic acid (OA), anisomycin, (R:(*)\), S:(*)-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidinepropanol (Ro 25-6981), staurosporine (STA) and 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) to produce stock solutions. The final
concentration of DMSO never exceeded 0.1% when applying these agents to slices; furthermore, control applications of DMSO 0.1% alone had no effect compared to baseline field recordings. Cyclosporin A (CyA) and (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (PEAQX) were dissolved in ethanol.

The majority of drugs were obtained from Tocris Cookson Ltd, analar grade compounds were obtained from BDH chemicals Ltd. UK. NMDA was obtained from Sigma chemical Co. Ltd. Ro 25-6981 was a gift from Dr Georg Jaeschke of F. Hoffmann-LaRoche AG Roche pharmaceuticals and PEAQX was a gift from Dr Yves Auberson of Novartis Pharmaceuticals (table 4).

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<tr>
<th>Compound</th>
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<tr>
<td>PEAQX</td>
<td>Novartis Pharmaceuticals</td>
<td>Ethanol</td>
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Table 4. List of pharmacological agents, suppliers and solvents used to produce stock solution.
Chapter 3. The negative coupling of NR2B to NR2A subunit-containing receptors.

Introduction

There has been a considerable amount of research over the last 10 years that has identified the NR2B subunit as being especially important in determining the function of the NMDA receptor. For example, in forming the glutamate binding site (Laube et al., 1997), controlling the Mg$^{2+}$ block (Williams et al., 1998) and in a variety of phenomena including learning and memory formation, synaptic plasticity (Hrabetova et al., 2000), pain (Chizh et al., 2001), schizophrenia (Grimwood et al., 1999; Gao et al., 2000), Parkinson’s disease (Nash et al., 1999; Oh et al., 1999; Dunah et al., 2000; Steece Collier et al., 2000), Huntington’s disease (Chen et al., 1999; Zeron et al., 2001) and excitotoxicity (Zhang et al., 1997; Cheng et al., 1999). The NR2B subunit consists of 1456 amino acids with a molecular weight of approximately 170-180 kDa. All of the NR2 subunits have the same basic structure as the NR1 subunit (Hollmann & Heinemann, 1994) but varying in that they have an especially large intracellular C-terminal domain. This C-terminal domain, it has been suggested, plays a role as the target for modulatory or accessory proteins in promoting receptor assembly, sorting or targeting and playing a part in different channel conformations to modulate receptor function (Kohr et al., 2003; Loftis &
Janowsky, 2003). The NR2B subunit is modulated by its co-assembly with other subunits, alterations in the interaction with associated proteins such as PSD-95 and SAP102, proteases, growth factors, hormones and post-translational modifications such as phosphorylation. The phosphorylation of tyrosine residues can contribute to enhanced receptor responses and signal transduction into the neurone (Gurd, 1985; Gurd & Bissoon, 1997; Hisatsune et al., 1999; Manabe et al., 2000; Nakazawa et al., 2001). Receptor function is also modulated by the phosphorylation of serine residues by protein kinase A (PKA), protein kinase C (PKC), and calmodulin-dependent protein kinase II (CaMKII) (Smart, 1997).

Ro 25-6981 is an ifenprodil derivative that has high affinity and selectivity for the NR2B subunit-containing NMDA receptor but without the affinity for α1-adrenergic and serotonergic receptors found with ifenprodil itself (Chenard et al., 1991; McCool & Lovinger, 1995). It is a high affinity, selective (5000-fold greater for the NR2B than the NR2A subunit), activity-dependent, voltage-independent and non-competitive NR2B subunit-containing NMDA receptor antagonist (Fischer et al., 1997; Mutel et al., 1998; Lynch et al., 2001).

F. Hoffman-La Roche Pharmaceuticals first described Ro 25-6981 in 1997. They used cloned NMDA receptors expressing NR1c/NR2A, NR1c/NR2B and NR1f/NR2B subunits to confirm that Ro 25-6981 was selective for the NR2B subunit-containing receptors. They also studied the
electrophysiology of Ro 25-6981 in young cultured rat cortical neurones, which express NR2B as the dominant NMDA receptor subunit. It was observed that the effect of Ro 25-6981 was dependent upon the relative level of NMDA receptor activation. Ro 25-6981 potently blocked the current evoked by NMDA 100μM but it potentiated the slight responses to NMDA 1μM. Protons (H+) exert a tonic inhibition of the NMDA receptor with an IC$_{50}$ at physiological pH 7.4 by binding at a proton sensor site. The NR2A and NR2B subunit-containing NMDA receptors are the most sensitive to this effect. The proton sensor’s binding site overlaps structurally and mechanistically with the binding site of Ro 25-6981, ifenprodil and the endogenous modulators spermine and spermidine (Mott et al., 1998). The binding stabilises a protonated, agonist-bound state of the receptor that has a low open probability. This shifts the pKa of the proton sensor to values that are more alkaline and increases tonic inhibition. However, the potentiation of responses to NMDA by these compounds is reported to be due to an allosteric modulation with the glutamate and glycine binding sites. The binding of these compounds causes a decrease in NMDA receptor affinity for glycine and an increase in affinity for glutamate and NMDA leading to potentiation. However, while ifenprodil and Ro 25-6981 both bind to the N-terminal third of the NR2B receptor, Ro 25-6981 has a distinct binding site that has greater three-dimensional requirements. Furthermore, the binding of ifenprodil is
decreased and its IC$_{50}$ is increased by rising concentrations of spermidine whilst under the same conditions the binding of Ro 25-6981 is unaffected and its IC$_{50}$ decreases (Kew et al., 1996; Kew et al., 1998b; Grimwood et al., 2000; Lynch et al., 2001; Perin-Dureau et al., 2002). In addition, Ro 25-6981 had no direct effect on the ion channel as seen with ifenprodil. This clearly demonstrates distinct properties of action by Ro 25-6981, which have not yet been fully investigated. This activity-dependent property is of great significance in therapeutic development as it predicts that NMDA receptors that are pathologically and continuously activated will be blocked whilst leaving inactive receptors relatively unaffected. In neuroprotective experiments, Ro 25-6981 was found to be very effective in protecting cultured cortical neurones from excitotoxic and ischaemic insults (Fischer et al., 1997).

A further study followed, using autoradiography to investigate the binding of Ro 25-6981 to adult rat brain membranes and tissue sections. It was observed that Ro 25-6981 binding was similar to NR2B subunit distribution and dissimilar to NR2A subunit distribution (Mutel et al., 1998).

More recently, a study of Ro 25-6981 was carried out to characterise its NR2B selective pharmacology using human embryonic kidney (HEK) 293 cells transfected with heterodimers consisting of NR1a and either wildtype, chimeric or mutant NR2B subunits (Lynch et al., 2001) which co-assemble
into tetrameric or pentameric receptors. It was observed that specific regions of the N-terminal leucine/isoleucine/valine-binding protein (LIVBP)-like domain of the NR2B subunit protein modulated the interaction between Ro 25-6981 and the NMDA receptor. Ro 25-6981 may be binding directly to the NR2B subunit or indirectly to the NR1 subunit at a site that is modulated by the NR2B subunit in a way that is analogous to glycine binding to the NR1 subunit where the NR2 subunit influences glycine affinity (Gallagher et al., 1996; Masuko et al., 1999; Perin-Dureau et al., 2002). Whilst Ro 25-6981 is an ifenprodil analogue it has a binding site and pharmacology that is distinct from the polyamine site (Lynch et al., 2001).

Although there are several NR2B antagonists available, Ro 25-6981 is the first compound to be developed that is a highly selective and full antagonist at NR2B subunit-containing NMDA receptors. Ifenprodil is a partial antagonist only inhibiting NR1/NR2B receptors by 80% on average and affecting NR1/NR2A/NR2B receptors with little (Luo et al., 1997) or intermediate effect (Tovar & Westbrook, 1999). Also, ifenprodil is not very selective for the NR2B subunit (400-fold, recombinant NR1/NR2B compared to NR1/NR2A) and also blocks α1-adrenergic, 5HT1A, 5HT2, 5HT3 and sigma receptors and N, P and Q type calcium channels (Chenard et al., 1991; McCool & Lovinger, 1995). Interestingly, Pfizer has recently developed an NR2B subunit-selective antagonist CP-101, 606, which has a
very low affinity for the NR1/NR2A/NR2B synaptic receptors but a high affinity for the purely extrasynaptic NR1/NR2B population (Chenard et al., 1995; Tovar & Westbrook, 1999). It would be of particular interest to repeat this study using CP-101, 606 to investigate further the contribution of synaptic versus extrasynaptic receptor populations (Hardingham et al., 2002). There have been several recent reports demonstrating that the different subunit-containing NMDA receptors and the locus of activation has a great effect on determining the direction of synaptic plasticity (Liu et al., 2004; Massey et al., 2004; Rusakov et al., 2004; Wong et al., 2004; Mallon et al., 2005) and the phosphorylation of CREB (cAMP response element binding protein) leading to BDNF expression and pro-survival pathways (Hardingham et al., 2002; Riccio & Ginty, 2002).

Novartis Pharmaceuticals have recently developed an NR2A subunit-containing NMDA receptor antagonist (R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-[(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (PEAQX, also known as NVP-AAM077, appendix 2). PEAQX is a water-soluble 5-methyl-quinoxalinedione with a high affinity for the glutamate-binding site of the NMDA receptor ([3H]-CGP39653 binding assay: IC$_{50}$ = 11nM). At 10μM PEAQX showed no additional activity in a series of 68 radioligand binding assays for CNS receptors. PEAQX has 126-fold selectivity for the NMDA NR1A/NR2A over the NR1A/NR2B receptors expressed in Xenopus oocytes (IC50 = 14nM and
1800nM respectively). Experiments on embryonic cortical cells and on neocortical slices have confirmed these results with native receptors. Cultured embryonic cortical cells express NR2A subunits at a very low level, resulting in a low potency of PEAQX ($IC_{50} = 1\mu M$). In adult slices where NR2A subunits are predominantly expressed the effect of PEAQX is stronger ($IC_{50} = 68nM$) than in cultured embryonic cortical cells that express NR2A at very low levels (undetectable) ($IC_{50} = 1\mu M$). Furthermore, in vivo PEAQX was found to be a potent anticonvulsant following intravenous dosing with few of the side effects associated with non-selective or NR2B selective antagonists (Auberson et al., 2002).

Although these compounds have been examined on recombinant receptors and immature cultures, which are systems that over-express the NR2B subunit-containing NMDA receptors, this study has used these compounds to examine the role of NR2 subunits in an intact neuronal network in which adult heteromeric NMDA receptors are involved in synaptic transmission and plasticity. Of great importance in this study was the use of adult animals that are 7-8 weeks old because they are past the 3rd postnatal-week threshold for the maturation of NMDA receptor subunit expression (where NR2A subunits are the last to reach their mature levels) and the 4th postnatal-week threshold during which the basal levels of receptor tyrosine phosphorylation are attained (Cudmore & Gurd, 1991; Tovar & Westbrook, 1999).
Results

NMDA

We started this study using 10-minute applications of NMDA, which reversibly depressed the slope of the fEPSP. The depression of fEPSP slope being a reflection of changes in membrane conductance and depolarisation of the neurones. This leads to the depolarisation block of the CA1 neurones recorded from and the subsequent blockade of excitatory transmission. The concentration-response relationship was very steep, with 1μM having had no effect, 4μM producing a response that was discernible but not statistically significant, while 7.5μM produced a depression to 69.36±16.33%, 10μM produced a depression to 15.18±4.43% (Figure 5) and 20μM producing a depression to -1.05±1.61% of fEPSP slope (Figure 4). Furthermore, investigation of changes to the paired-pulse paradigm during the depression elicited by NMDA 10μM found a significant decrease of paired-pulse inhibition, and increase of paired-pulse facilitation at all interstimulus intervals (10ms p<0.001, 20ms, p<0.01 and 50ms p<0.01, n=5) (Figure 6 and 7).
Figure 4. Concentration curve of the inhibitory effect of NMDA upon fEPSP slope.

Graph showing the non-cumulative concentration-response curve of depression of fEPSP slope in response to 10-minute applications of NMDA concentrations of 1μM, 4μM, 7.5μM, 10μM and 20μM, n=4, 13, 4, 8 and 4 respectively. A concentration of 8.6μM is predicted to cause a 50% depression of fEPSP slope. Each point shows the mean±s.e.m.
Figure 5. Effect of NMDA 10μM on fEPSP slope.

Plot showing the effect of a 10-minute application of 10μM NMDA, which elicits a depression in the slope to 15.18±4.43% of fEPSP (p<0.001). Points (c), (1) and (2) indicate the timing of the paired-pulse tests summarised in Figure 6. Each point shows the mean±s.e.m. for n=8 experiments.
Figure 6. Effect of NMDA 10μM on paired-pulse interactions.

Graph showing the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms during the baseline control responses (c), 10μM NMDA (1) and the washout period (2) (10ms p<0.001, 20ms p<0.01, 50ms p<0.01, n=5, 1 compared to 2 and c)(see plot in figure 5 for corresponding times points and example records in figure 7). Each point shows the mean±s.e.m. for n=5 experiments.
Figure 7. Example records of paired-pulse interactions during baseline recording and under the influence of 10µM NMDA.

Showing paired-pulse interactions recorded during baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression caused by NMDA 10µM: (d) 10ms (e) 20ms and (f) 50ms (points relate to same points in figures 5 and 6).
Ro 25-6981

The large depressions of fEPSP slope elicited by applications of NMDA were caused by the activation of native heteromeric NMDA receptors containing a combination of NR1, NR2A, NR2B and NR2D subunits. This study investigated the contribution of NR2B subunit-containing NMDA receptors using the NR2B antagonist Ro 25-6981. A concentration of 3μM Ro 25-6981 was chosen as it has previously been shown to block selectively the NR2B component of the NMDA excitatory postsynaptic current (NMDAEPSC) in 3-4 week old rats and not the NR2A component (Liu et al., 2004). Repeated applications of NMDA 10μM caused two similar reversible depressions of fEPSP slope to 16.06±9.16% then to 12.85±7.85% (p<0.001, n=4). The co-application of 3μM Ro 25-6981 had no immediate effect upon this depression, which again reversibly depressed the fEPSP slope to 8.96±2.77% (p<0.001, n=4). However, the next 10-minute application of NMDA started 5 minutes after the end of the 20-minute application of Ro 25-6981 3μM totally abolished the fEPSP, which then slowly and partially recovered to 37.56±7.26% of the baseline responses and remained depressed for at least the 100 minutes recorded (p<0.001, n=4) (figure 8 and 9). Attempts to recover the response by increasing afferent stimulation or by HFS stimulation, which normally induces LTP (responses not shown), were unsuccessful. This observation is consistent with a terminal excitotoxic insult to the neurones recorded
from and inconsistent with the induction of LTD as witnessed by the failure to reverse it with an LTP inducing protocol or by increasing the stimulation of the presynaptic afferents (Schurr et al., 1995).

Following the intensity of the response to 10μM NMDA, a concentration of NMDA 4μM was used, a concentration that produced a smaller control depression of baseline fEPSP slope (figure 10). This would be less likely to result in an excitotoxic insult after the application of Ro 25-6981. Applications of NMDA 4μM caused two small yet discernible depressions of fEPSP slope that were not statistically significant (i and ii). Again the co-application of Ro 25-6981 3μM with a third application of NMDA (iii) had no immediate effect but a fourth application, during the washout phase of Ro 25-6981 (iv), showed a significant enhancement of the depression of fEPSP slope. The fifth application of NMDA (v) caused an even greater depression of fEPSP slope that was matched in size by the sixth and seventh (vi and vii) applications with no washout of the effect being observed up to 100 minutes after Ro 25-6981 had been removed from the perfusate (significant depressions of fEPSP slope to (iv) 40.94±4.56%, (v) 19.78±6.7%, (vi) 19.28±10.74% and (vii) 13.23±4.18% (all p<0.001 compared to the second NMDA 4μM induced depression, n=4)). Lastly, the application of the NR2A subunit-selective antagonist PEAQX 0.1μM during the eighth and final application of NMDA 4μM (viii) completely
blocked any depression of fEPSP slope i.e. both the initial control NMDA 4μM portion and the enhanced portion induced by Ro 25-6981 (figure 10).

This study also investigated the effect of Ro 25-6981 upon responses to low concentrations of NMDA using intracellular recording techniques to investigate the effect solely on the excitability of the postsynaptic pyramidal cell (Appendix 1). A similar enhancement of responses to NMDA was induced over the same time course as seen with extracellular recordings. This suggests a predominantly postsynaptic site of action for these effects.
Figure 8. Repeated 10-minute applications of 10μM NMDA and the effect of 3μM Ro 25-6981.

Plot showing the co-application of 3μM Ro 25-6981 had no immediate effect upon the response to 10μM NMDA, whereas the next application of NMDA was potentiated to the extent that the fEPSP was initially abolished and then shows only partial recovery ($p<0.001$, $n=4$). Each point shows the mean±s.e.m. for $n=4$ experiments.
Figure 9. Representative fEPSPs showing excitotoxic damage.

Recordings of fEPSPs showing the irrecoverable depression of baseline responses caused by the excitotoxic insult of NMDA 10µM and Ro 25-6981 3µM (2) compared with baseline responses (1). Numbered traces correspond to the time course in figure 8.
Figure 10. Effect of Ro 25-6981 and NMDA 4\mu M on fEPSP slope.

Plot showing the effect of repeated 10-minute applications of 4\mu M NMDA, which produced a small depression of the fEPSP slope. The application of 3\mu M Ro 25-6981 had no immediate effect upon this, but subsequent applications of 4\mu M NMDA show the development of potentiation in the fEPSP slope (p<0.001, n=4). The co-application of PEAQX at 0.1\mu M completely blocked the final potentiated response to NMDA. Each point shows the mean±s.e.m. for n=4 experiments.
NMDA receptor antagonism

A surprising feature of these experiments was that the effect of Ro 25-6981 developed slowly, required at least 20 minutes to elicit a clearly significant decrease in response size and the effect gradually continued to develop during the washout phase. In addition this effect, once fully developed, was long lasting, with no attenuation of the depressed response size being observed for up to 100 minutes post-washout. The continuous bath-application of NMDA 4μM was employed in order to observe better how the effect of Ro 25-6981 developed. As shown in figure 11, NMDA 4μM initially produced a small, stable depression of baseline fEPSP slope, the co-perfusion of Ro 25-6981 3μM produced a slight recovery of the responses but near the end of the 20-minute perfusion the depression gradually develops. As Ro 25-6981 started to washout the depression had reached 70.42±12.18% of baseline fEPSP slope. In spite of the absence of perfusing Ro 25-6981 the depression continued to gradually develop. The depression stabilised 30 minutes after the washout of Ro 25-6981 at 33.84±0.32% (p<0.001; n=5) of baseline fEPSP slope. The application of the competitive, non-selective NMDA receptor antagonist 2-AP5 50μM reversed the depression to near-baseline responses. However, after the washout of 2-AP5 the depression reasserted itself within approximately 18 minutes (p<0.001; n=5). At the end of the experiment and with the washout of NMDA, the fEPSP slope returned to a level not significantly
different from the initial baseline responses (Figure 11). An examination of paired-pulse interactions during this experiment found that paired-pulse ratios were enhanced during the Ro 25-6981-mediated enhancement of NMDA 4μM (p<0.001, n=5 at all interstimulus intervals) but were returned to baseline responses during the reversal of this depression caused by 2-AP5 (figure 12).

Repeating the same protocol using a slightly higher level of NMDA at a concentration of 7.5μM (figure 13) produced a modest and stable depression of fEPSP slope to 64.6±10.4% (not significant, n=4). The co-application of Ro 25-6981 3μM caused an enhancement of this depression, which started during Ro 25-6981 co-perfusion and continued during washout until it reached a stable plateau of -0.16±0.24% (p<0.01; n=4) of baseline fEPSP slope within approximately 18 minutes of the depression beginning. The application of the competitive, non-selective NMDA receptor antagonist 2-AP5 50μM significantly reversed the depression of fEPSP slope back to the level previously seen with NMDA alone (p<0.01; n=4). Again, during the washout of 2-AP5 the depression reasserted itself within approximately 18 minutes to 0.80±0.29% (p<0.001; n=4). At the end of the experiment and with the washout of the NMDA, the fEPSP returned to a level not significantly different from the baseline responses although it was apparent that the level and duration of NMDA agonism had had a deleterious effect upon the response size. The rate of onset of effect
was quicker with NMDA 7.5µM (18 minutes) than with NMDA 4µM (30 minutes) perhaps reflecting the increased rate of binding with increased concentration of NMDA as previously reported with glutamate (Mutel et al., 1998) and with ifenprodil (Grimwood et al., 2000). Figure 14 shows a concentration-response curve of the effect of NMDA concentrations and the degree to which they are potentiated to under the influence of Ro 25-6981 3µM. This shows a shift to the left of the NMDA concentration-response curve after the application of Ro 25-6981. The concentration required to elicit a 50% depression of fEPSP slope changed from 1.7µM with NMDA alone to 8.6µM under the influence of Ro 25-6981.
4μM NMDA caused a slight depression in the fEPSP slope (c). The co-application of 3μM Ro 25-6981 increased this response; the increase occurred slowly during Ro 25-6981 washout into a stable plateau, (1) fEPSP slope of 33.84±0.32% (p<0.001; n=5) compared with the baseline. The competitive NMDA receptor antagonist 2-AP5 (50μM) reversed the depression (2) (p<0.001; n=5). During the washout of 2-AP5, the potentiated response to NMDA re-asserted itself (p<0.001; n=5), but recovered to baseline values upon removing the NMDA. Each point shows the mean±s.e.m. for n=5 experiments.
Figure 12. Effect of NMDA 4μM and Ro 25-6981 on paired-pulse interactions.

This graph summarises paired-pulse interactions at interstimulus intervals of 10 ms, 20 ms and 50 ms, corresponding to points (c), (1) and (2) in figure 11. The results demonstrate a significant enhancement of paired-pulse interactions during the trough of the depression caused by Ro 25-6981 and NMDA (1) (10 ms p<0.001, 20 ms p<0.001, 50 ms p<0.001, n=5) compared with baseline responses (c). These changes were reversed by 2-AP5 (2). Each point shows the mean±s.e.m. for n=5 experiments.
Figure 13. Effect of Ro 25-6981 on the continuous application of NMDA 7.5μM and its reversal by 2-AP5.

7.5μM NMDA caused a modest (64.6±10.4%; n.s. n=4) depression of the fEPSP slope. The co-application of 3μM Ro 25-6981 increased this response; the increase occurred slowly during Ro 25-6981 washout into a stable plateau fEPSP slope of -0.16±0.24% (p<0.01; n=4) compared with the baseline. The competitive NMDA receptor antagonist 2-AP5 (50μM) reversed the depression (p<0.01; n=4). During the washout of 2-AP5, the potentiated response to NMDA re-asserted itself (p<0.001; n=4), but recovered to baseline values upon removing the NMDA. Each point shows the mean±s.e.m. for n=4 experiments.
Figure 14. The concentration-response curve of NMDA alone and after Ro 25-6981.

Graph summarising the concentration-response curves for responses to NMDA in the presence and absence of 3μM Ro 25-6981, and showing the marked leftward shift produced by the combination. NMDA alone (1, 4, 7.5, 10 and 20μM) and Ro 25-6981 potentiated responses to NMDA (0.1, 1, 4, 7.5 and 10μM). The concentration eliciting a 50% depression of fEPSP slope changes from 8.6μM for NMDA alone to 1.7μM under the influence of Ro 25-6981. Each point shows the mean±s.e.m.
2-AP5 is a competitive antagonist at the NMDA receptor’s glutamate binding site, in order to test that this was not a compounding factor in the blockade of the effect of Ro 25-6981 the non-competitive, open channel blocker dizocilpine (MK 801) was used to antagonise the NMDA receptor during the co-application of Ro 25-6981 and NMDA. The application of NMDA 4μM caused a small, stable depression of fEPSP slope that was reversed by the application of MK 801 10μM to baseline response size. The perfusion of Ro 25-6981 3μM had no effect upon the fEPSP slope and during the washout of MK 801 the response size remained at baseline levels (figure 15). However, the non-competitive antagonism provided by MK 801 was not washed out of the slice over the time-period recorded from and this is a likely reason for the lack of any depression observed.
Figure 15. Effect of 4μM NMDA and Ro 25-6981 in the presence of MK 801 on fEPSP slope.

Plot showing the effect of 10μM MK 801 applied throughout the combined application of 4μM NMDA and 3μM Ro 25-6981. An initial, small depression (approximately 5%) produced by NMDA was blocked by 10μM MK 801. This prevents the development of a potentiated response to NMDA plus Ro 25-6981 even after the washout of MK 801. Each point is the mean±s.e.m. for n=3.
Having previously demonstrated that the Ro 25-6981-mediated potentiation of responses to NMDA was transiently reversible by applying 2-AP5 50μM this study investigated whether, by applying this competitive antagonist before, during and after Ro 25-6981, the induction of the depression of fEPSP slope could be prevented. As shown in figure 16 the application of NMDA 4μM produced a slight, stable depression of the fEPSP slope that was reversed to above the baseline levels by the subsequent application of 2-AP5. The co-application of Ro 25-6981 3μM had no further effect upon the fEPSP response, 2-AP5 preventing the depression normally elicited. However, as 2-AP5 was washed out the depression of fEPSP slope developed rapidly to 14.06±5.09% of baseline fEPSP slope (p=0.0178, n=4) within approximately 20 minutes of it starting to reduce. The gradient of the development of this depression was similar to that seen with 2-AP5 washout in figure 11 and 13; i.e. a depression that is linear and rate-dependent upon the washout of 2-AP5. The gradient was dissimilar to the Ro 25-6981/NMDA 4μM gradient of figure 11, which is rate-dependent on the effect initiated by Ro 25-6981, which is a slower developing effect (approximately 30 minutes). Therefore, the effect of Ro 25-6981 would seem to have already been realized during 2-AP5 co-perfusion suggesting that it does not require NMDA receptor activation. The difference in these gradients may be explained by the binding of Ro 25-6981 having already taken place during 2-AP5 perfusion; 2-AP5 was able to suppress the NR2A
activation-dependent expression of its effect but not the binding of Ro 25-6981 to its unique site on the NR2B subunit and the mechanism it puts into effect to produce the enhanced depression of fEPSP slope.
Figure 16. Effect of 2-AP5 continuously bath-applied during the application of 4μM NMDA and Ro 25-6981 on fEPSP slope.

A plot showing the effect of 50μM 2-AP5 applied throughout the combined application of 4μM NMDA and 3μM Ro 25-6981. An initial, small depression (approximately 5%) produced by NMDA was blocked by 50μM 2-AP5. Despite this, the application of 2-AP5 did not prevent the development of a potentiated response to NMDA plus Ro 25-6981, which appeared after washout of the 2-AP5 and persisted until the NMDA was removed. Each point is the mean±s.e.m. for n=4 experiments.
The bath application of NMDA in the adult rat hippocampus will activate NMDA receptors containing NR2A, NR2B and NR2D subunits. In view of the potentiating activity produced when the NR2B element to the action of NMDA was blocked by Ro 25-6981, the putative NR2A subunit antagonist PEAQX was used to investigate the residual response to NMDA. NMDA 4\mu M was continuously bath-applied producing a small, stable depression of fEPSP slope (figure 17). The co-perfusion of 3\mu M Ro 25-6981 transformed this into a large depression to 31.90\pm1.01\% of the baseline fEPSP slope (p<0.05, n=3) that developed over approximately 30 minutes. It was then reversed by the application of PEAQX 0.1\mu M back to baseline fEPSP responses. The rate of washout of PEAQX was initially rapid but after 8 minutes, it slowed to such an extent that it still did not completely washout for at least 2 hours. These experiments were therefore concluded before the depression might be observed to fully re-establish.
Figure 17. Effect of NMDA, Ro 25-6981 and PEAQX on fEPSP slope.

This plot shows the interaction of NMDA, Ro 25-6981 and PEAQX. 4μM NMDA produces a small, stable depression, but co-perfusion of 3μM Ro 25-6981 transforms this into a large depression that reaches 31.90±1.01% of the baseline fEPSP slope (p<0.05, n=3). This depression was reversed by 0.1 μM PEAQX. The partial washout of PEAQX stabilised after 8 minutes but does not completely wash out for at least two hours. The experiment was therefore terminated before the depression was fully re-established. Each point is the mean±s.e.m. for n=3 experiments.
Protein phosphorylation

The phosphorylation state of the NMDA receptor and many other cellular proteins determines their activity and function (Smart, 1997). This study next investigated the involvement of protein kinases and protein phosphatases on the Ro 25-6981 effect.

Staurosporine (STA) is a broad-spectrum protein kinase inhibitor, enzymes inhibited include: PKC (IC\textsubscript{50}=3nM), PKA (IC\textsubscript{50}=7nM), tyrosine protein kinase (IC\textsubscript{50}=6nM) and CaMKII (IC\textsubscript{50}=20nM) (Tamaoki et al., 1986; Ruegg & Burgess, 1989; Yanagihara et al., 1991). As shown in figure 18 the initial 10-minute application of NMDA 4\mu M caused a small depression of the fEPSP slope, staurosporine 100nM was continuously bath-applied and had no effect upon baseline fEPSP slope or the depression produced by a further 10-minute application of NMDA 4\mu M. The application of Ro 25-6981 3\mu M and the subsequent application of NMDA produced a significant depression of fEPSP slope to 17.66±10.83\% (p<0.01, n=3) compared to baseline responses. After washout, a final application of NMDA 4\mu M produced another significant depression of fEPSP slope to 1.77±0.87\% (p<0.001, n=3) compared to baseline responses (Figure 18). Therefore, staurosporine was unable to prevent the potentiation of responses to NMDA 4\mu M by Ro 25-6981.
The first phosphatase inhibitor investigated was okadaic acid (OA), a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A (PP1 and PP2A) (Bialojan & Takai, 1988). The continuous application of NMDA 4μM elicited a small, stable depression of fEPSP slope. The addition of okadaic acid before, during and after the co-application of Ro 25-6981 3μM was unable to prevent the development of a significant depression of the fEPSP slope to 1.56±0.31% (p<0.001, n=4). Interestingly, upon washout the fEPSP slope recovered to an average of 156.5±14.62% (p<0.05, n=4) of baseline responses, a significant LTP of responses that was stable (figure 19). This LTP-inducing effect was not investigated further at this time.

Cyclosporin A (CyA), a widely used serine/threonine Ca²⁺/calmodulin-dependent phosphatase 2B (calcineurin) inhibitor, was used next. The continuous bath-application of NMDA 4μM again produced a slight, stable depression of fEPSP slope. The application of 1μM cyclosporin A had no significant effect on the baseline fEPSP slope but it was able to prevent the anticipated enhancement of the NMDA 4μM-mediated depression of fEPSP slope previously seen after the application of Ro 25-6981 3μM (figure 20). Interestingly the baseline fEPSP returned to values that were significantly potentiated (132±0.81%, p<0.05, n=4) compared to baseline responses. The application of cyclosporin A prevented the depression of fEPSP slope seen with both the control group and the experiments using
okadaic acid and staurosporine, which were unable to inhibit the development of the depression. Interestingly, the magnitude of the depression of fEPSP slope was significantly greater than the control group for both okadaic acid and staurosporine (figure 21).
Figure 18. Action of staurosporine upon the effect of the co-application of 4μM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing repeated applications of NMDA 4μM produced a small depression of fEPSP slope that was not prevented by the co-application of 100nM staurosporine. The co-application of staurosporine was also not able to prevent the enhancement of NMDA responses by Ro 25-6981 3μM which produced a significant depression of the fEPSP slope to 17.66±10.83% (p<0.01). Each point shows the mean±s.e.m. for n=3 experiments.

100
Figure 19. Action of okadaic acid upon the effect of the co-application of 4μM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing that the continuous application of NMDA 4μM produced a small depression of fEPSP slope. The co-application of staurosporine before, during and after Ro 25-6981 was not able to prevent the enhancement of NMDA responses by Ro 25-6981 3μM which produced a significant depression of the fEPSP slope to 1.56±0.31% (p<0.001, n=4). Each point shows the mean±s.e.m. for n=4 experiments.
Figure 20. Prevention by cyclosporin A of the effect of the co-application of 4μM NMDA and Ro 25-6981 on fEPSP slope.

Plot showing the effect of 1μM cyclosporin A (CyA) upon the Ro 25-6981 potentiation of NMDA responses. 4μM NMDA produced a slight, non-significant depression of fEPSP slope; the application of CyA had no significant effect on this but prevented the enhancement of the NMDA response by 3μM Ro 25-6981. Each point shows the mean±s.e.m. for n=4 experiments.
Figure 21. Column bar graph summary of phosphorylation modulators.

This histogram summarises the depression caused by the co-application of 4μM NMDA and 3μM Ro 25-6981 under control conditions (figure 11, n=4) and with the co-perfusion of 1μM cyclosporin A (CyA) (figure 20, n=4), 50nM okadaic acid (OA) (figure 19, n=4) and 100nM staurosporine (STA) (figure 18, n=3). Cyclosporin A prevented the depression of the fEPSP slope induced by NMDA in the control group (p<0.001). However, okadaic acid and staurosporine increased the magnitude of the depression (both p<0.01).
Protein synthesis

In view of the latency of onset, the long-lasting effect of Ro 25-6981, and the involvement of protein phosphorylation the question arose of whether any permanent change was required and induced of new protein synthesis. To investigate this possibility anisomycin, a widely used reversible translation inhibitor was used (Grollman, 1967). The application of NMDA 4μM produced a small, stable depression of fEPSP slope. The continuous application of anisomycin 30μM had no significant effect upon the fEPSP slope nor was it able to interfere with the subsequent NMDA and Ro 25-6981-mediated depression of fEPSP slope to 10.65±0.3628% (p=0.0128, n=4)(figure 22).
Figure 22. Effect of anisomycin on the Ro 25-6981/NMDA effect upon fEPSP slope.

Plot showing that 30μM anisomycin was unable to prevent the development of the Ro 25-6981-mediated enhancement of NMDA responses (p=0.0128, n=4). Each point shows the mean±s.e.m. for n=4 experiments.
Discussion

Applications of NMDA produced a concentration dependent depression of evoked fEPSP slope that was associated with an enhancement of paired-pulse ratios at all interstimulus intervals. The fEPSP under normal 0.05Hz stimulation is predominantly a reflection of the fast kinetics of the AMPA receptors which underlie this recording, with fast rise times (10-90% rise time of ~400μs) and fast decay time constant (~1.3ms). The NMDA component, in contrast, has slow kinetics with a slow rise time (~9ms) and a slow mean decay time constant (~52ms) (Gibb et al., 1994). Schaffer collateral inputs onto CA1 neurones elicit fEPSPs where the NMDA receptors contribute only very a small component (~3%) (Arrigoni & Greene, 2004), principally as a result of the magnesium block of the channel at resting membrane potentials. At these negative membrane potentials, the magnesium ion blocks the ion channel in a voltage-dependent non-competitive manner. The depolarisation induced by a single fEPSP is not sufficient to overcome the threshold leading to NMDA receptor activation because the membrane potential does not reach a sufficient level to expel the magnesium ion. However, repetitive activation of the synapse such as that seen in HFS and LFS stimulation protocols to induce LTP and LTD produces a non-linear summating effect which overcomes this threshold and leads to the activation of the NMDA receptors. It is apparent from current-voltage plots for NMDA-induced
conductances that even at $-90\text{mV}$, although the NMDA current is very small it is never entirely prevented by magnesium block (Mayer et al., 1984; Nowak et al., 1984). The bath application of NMDA receptor agonists will lead to the activation of synaptic and extrasynaptic NMDA receptors which will result in large conductance through their 50pS channels over longer time periods than that seen with AMPA receptors, producing a regenerative feedback that leads to the unblocking of NMDA receptors and their activation. This conductance then leads to the depolarisation of the cells recorded from and the subsequent depolarisation block of excitatory synaptic transmission, manifested as a loss of the fEPSP slope. The washout of NMDA agonist allows the neurones to rebuild the ionic gradients that underlie the membrane potential and the fEPSP response recovers. The application of NMDA agonists which leads to this depolarisation will also lead to the activation of GABA$_A$ and GABA$_B$ receptors responsible for fast and slow components of feed-forward and feedback inhibitory transmission. Although this study did not address the contribution of these effects on the changes in the depression of fEPSP slope or on paired-pulse interactions previous investigations found that GABA, bath-applied to the hippocampal slice, elicited no inhibition of the fEPSP slope or altered the paired-pulse interactions in concentrations up to 1mM (unpublished observations). This suggested that the contribution of additional GABA release in this preparation is limited or already saturated by concentrations of GABA released in response to afferent stimulation.
Ro 25-6981 has been described as a selective antagonist at the NR2B subunit of the NMDA receptor. However, the previous work was carried out deliberately on immature tissue or cloned receptors overexpressing the NR2B subunit and not in adult tissues expressing mature NMDA receptors. When tested on the intrinsic neuronal circuitry of the adult hippocampal slice Ro 25-6981 unexpectedly potentiated NMDA receptor activation at all concentrations of NMDA tested and showed no inhibition of NMDA responses. The increased depression of fEPSP slope was generated through NR2A subunit-containing receptors, since the potentiated response could be blocked selectively by PEAQX. Furthermore, this enhancement appeared to have a presynaptic component, since the paired-pulse ratios were significantly enhanced during potentiated responses to NMDA at concentrations that previously showed no such change from baseline responses. It is widely recognised that a loss of paired-pulse inhibition and an increase of paired-pulse facilitation represents a presynaptic action of the test agents. Although it does not follow that postsynaptic effects cannot influence the appearance of PPD and PPF in field recordings (Seabrook et al., 1999). Additionally, evidence from intracellular recordings of the enhancement of NMDA responses by Ro 25-6981 suggests that this phenomenon has a predominantly postsynaptic locus. The presynaptic element of responses to NMDA suggested in this study may be mediated via NR2A or NR2A/NR2B subunit-containing receptors. This is consistent with evidence for the existence of presynaptic NMDA receptors, which
may be responsible for changes in presynaptic release probability and the changes in paired-pulse interactions described here.

The most parsimonious explanation of the present results would be that the NR2B subunits exert a tonic inhibitory restraint on the activation of NR2A subunit-containing NMDA receptors. Blockade of the NR2B subunits then results in disinhibition of NR2A subunit-containing receptors, which become the primary effectors of the increased response to NMDA. This would explain both the potentiating effect of Ro 25-6981 and the blockade of the potentiated responses by PEAQX. It is not possible to conclude from the present data whether this inhibitory subunit interaction occurs between subunits within single heterotrimeric NR1/NR2A/NR2B receptors or between discrete heterodimeric receptors containing exclusively NR1/NR2A and NR1/NR2B subunits. Additionally, the interaction may be between receptor populations located at different sites interacting by way of intracellular or retrograde messengers.

These results are partially consistent with reports that ifenprodil and Ro 25-6981 caused small inward currents produced by NMDA 1μM to be potentiated, while larger currents produced by NMDA 100μM were markedly inhibited (Kew et al., 1996; Fischer et al., 1997). However, no inhibition against any concentrations of NMDA in the intact neuronal network of the adult hippocampal slice was observed. This is most simply
explained by the very different expression of subunits and their phosphorylation within cloned receptor constructs, immature tissues and with the mature tissue used in this study but merits further investigation.

It also appears from the data in this study that the interaction between NR2B and NR2A subunits was not prevented by inhibitors of protein kinases or the serine/threonine protein phosphatases types 1 and 2A (PP1 and PP2A) (Bialojan & Takai, 1988). A finding consistent with our later observation that Ro 25-6981 was unable to block LTP induction, which requires the activation of protein kinases (Bliss & Collingridge, 1993). Although the interaction between NR2B and NR2A subunit-containing receptors was not prevented by the broad-spectrum protein kinase inhibitor staurosporine, it is nevertheless likely that constitutively active kinases are essential to phosphorylate the unknown moiety to maintain the inhibitory restraint described. The subunit interaction does appear to involve Ca²⁺/calmodulin-dependent phosphatase 2B (calcineurin), since it was prevented by cyclosporin A. Calcineurin has previously been shown to enhance both the presynaptic release of glutamate and postsynaptic responsiveness to it (Yakel, 1997) partly by altering channel gating properties and promoting desensitisation in cultured hippocampal neurons (Lieberman & Mody, 1994; Nichols et al., 1994; Sihra et al., 1995; Tong et al., 1995; Victor et al., 1995). Furthermore, the calcineurin inhibitors cyclosporin A and FK506 are able to prevent the induction of LTD
(Mulkey et al., 1994; Hodgkiss & Kelly, 1995). Interestingly, it has been reported that calmodulin-dependent inhibition of NMDA receptors will reduce the amplitude and time course of excitatory synaptic currents in 12-day-old rat hippocampal NMDA receptors (Rycroft & Gibb, 2002). NR2 subunits, unlike NR1 subunits, are known to be highly phosphorylated under basal conditions (Hall & Soderling, 1997), therefore the phosphorylation step described could be acting directly upon the NR2A and/or the NR2B subunit to produce the enhancement of responses to NMDA. It is however possible that the phosphorylation site is elsewhere on the NMDA receptor complex, which has been reported from proteomic analysis to associate with at least 77 proteins (Husi et al., 2000) e.g. NR1, PSD-95, SAP 102, or at a non-NMDA site.

The present work also reveals that the potentiating effect of Ro 25-6981 is not dependent upon new protein synthesis, although it is expected that relevant proteins are constitutively present. The reason for the slow onset and apparently irreversible nature of the enhancement effect remains unclear. It may be due for example to the Ro 25-6981 binding irreversibly, to a persistent modification of the NR2B, NR2A or NR1 subunit that is then independent of Ro 25-6981 binding, to an irreversible uncoupling of the NR2B subunit from NR2A or to a change in NMDA receptor functional expression. Radiolabelled forms of Ro 25-6981 are unavailable at this time to investigate the possibility of it binding irreversibly to the NR2B subunit.
Chapter 4. The NMDA subunit specificity of LTP

Introduction

It is widely accepted that a persistent change in synaptic strength is the cellular basis of learning and memory (Alkon & Nelson, 1990; Kandel, 1997). Both long-term potentiation (LTP) and long-term depression (LTD) have now been shown to fulfil three out of the four criteria that are required to verify that they are both ‘necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed’. Alterations in synaptic strength are detectable after learning, interfering with the induction and expression mechanisms results in concurrent interference in learning, and altering the pattern of synaptic weights after learning affects the ability of animals to remember a previously learned experience. The, unfulfilled, fourth criterion is mimicry, where an implanted pattern of synaptic weights generates in the animal the behaviour of a specific recalled memory (Moser et al., 1998).

LTP, first reported in the dentate gyrus (Lomo, 1966; Bliss & Lomo, 1973), has since been demonstrated in all three excitatory pathways in the hippocampus (Schwartzkroin & Wester, 1975; Alger & Teyler, 1976) and elsewhere in the CNS (Kuba & Kumamoto, 1990). LTP is characterised by input specificity, co-operativity and associativity, leading to the proposal that LTP is a Hebbian process (Hebb, 1946), where the co-incident activity
of pre- and postsynaptic neurones is required to increase persistently synaptic strength. LTP was first shown to be involved in the cellular basis of learning and memory with experiments to block LTP in vivo coincided with deficits in hippocampal-dependent place navigation (Morris et al., 1986). LTP induction in the CA1 area is NMDA receptor dependent: it is triggered by NMDA receptor activation and blocked by NMDA receptor antagonism (Collingridge et al., 1983). AMPA receptor-mediated depolarisation of the postsynaptic cell during LTP induction, by high frequency stimulation (HFS), releases the Mg$^{2+}$ block of NMDA receptors allowing Ca$^{2+}$ to flow into the cell (Lynch et al., 1983; Mayer et al., 1984; Nowak et al., 1984; Malinow & Miller, 1986; Wigstrom et al., 1986). A large rise in Ca$^{2+}$ in the postsynaptic spine sets off a cascade of events that results in LTP (Bliss & Collingridge, 1993). The induction of LTP is widely considered to be postsynaptic and the expression both pre- and postsynaptic (Kullmann & Nicoll, 1992; Manabe et al., 1993). The maintenance of LTP is characterised by an early phase which is dependent on post-translational modifications and lasts for a few hours, followed by later phases which are dependent on de novo gene expression and protein synthesis that lasts for many hours (Krug et al., 1984; Otani et al., 1989; Nguyen et al., 1994). LTP can persist for varying amounts of time dependent upon the induction protocol and the experimental model. It has recently been demonstrated that stable LTP can last for up to a year (Abraham et al., 2002).
There is much evidence that supports a role for the NR2B subunit in synaptic plasticity and specifically in LTP. Increases in the expression of the NR2B and not of the NR2A, NR2C or NR2D subunit after LTP induction and the prevention of LTP induction in an NR2B knock-down model support a role for the NR2B subunit in the early phase of LTP (Thomas et al., 1994; Thomas et al., 1996). Furthermore, enhanced tyrosine phosphorylation of the NR2B subunit has been demonstrated after the induction of LTP (Rosenblum et al., 1996; Manabe et al., 2000; Nakazawa et al., 2001). LTP and LTD are bi-directional mechanisms of synaptic plasticity, induced by different patterns of afferent stimulation (Dudek & Bear, 1993). A recent investigation was made into whether these different patterns reflect the activation of specific subunits to produce either LTD or LTP. It was found that antagonists with a higher affinity for the NR2A/B subunits over the NR2C/D subunits produced greater inhibition of LTP than LTD induction (Hrabetova et al., 2000). There are also reports that the NR2B subunit is associated with age-related changes (Magnusson, 2000), the decrease in NR2B expression with increasing age leading to the blockage of LTP induction and impairments in spatial learning performance (Clayton et al., 2002). Potentiating the NMDA receptors using spermine, an endogenous polyamine, could reverse this. Taking advantage of the new NR2B and NR2A subunit-selective antagonists, Ro 25-6981 and PEAQX, this investigation sought to determine the subunit specificity of NMDA-dependent LTP induction.
Results

LTP was induced by high frequency stimulation (3 identical trains of 1 second stimulation bursts at 100Hz 10 minutes apart; HFS). This produced an enhancement of fEPSP slope to 197.9±16.4% (p<0.001, n=6) that persisted steadily for 140 minutes from the start of the induction protocol (figure 23 and 24).

To demonstrate that the LTP induced by HFS was NMDA receptor-dependent 50μM 2-AP5 was applied during the induction protocol and prevented LTP induction (figure 25). PEAQX 0.1μM was then used, which had previously been seen in chapter 3 to block NMDA responses in an analogous manner to 2-AP5. The perfusion of this NR2A antagonist also prevented the induction of LTP (figure 26). Finally, the NR2B antagonist Ro 25-6981 was used but it was unable to prevent the induction of LTP to 170.4±15.7% (p<0.01, n=6) of baseline fEPSP slope, which was less than control HFS induced LTP but not significantly so (figure 27).

This result clearly demonstrates that the NR2A subunit, and not the NR2B subunit, is critically required for the induction of NMDA-dependent LTP (figure 28).
100Hz stimulation of 1 second duration.

Figure 23. Effect of high frequency stimulation on fEPSP slope.

Plot showing LTP induced by three trains of stimuli using 100 pulses at 100 Hz, ten minutes apart (HFS) at 20, 30 and 40 minute time points, producing a persistent enhancement of the fEPSP slope to 197.9±16.4% of the control size ($p<0.001$, $n=6$). Each point shows the mean±s.e.m. for $n=6$ experiments.
Figure 24. Representative fEPSPs demonstrating LTP.

Illustrating baseline responses (c) and potentiated responses (1) as a result of LTP induction; points correspond to figure 23.
Figure 25. Blockade of NMDA-dependent LTP induction by 2-AP5.

Plot showing the perfusion of 2-AP5 50μM during the HFS induction protocol for LTP which prevented the induction of LTP, n=3. Each point shows the mean±s.e.m. for n=3 experiments.
Figure 26. Blockade of NMDA-dependent LTP induction by PEAQX.

Plot showing the effect of PEAQX 0.1 μM upon the induction of LTP. The perfusion of PEAQX prevented the induction of LTP. Each point shows the mean ± s.e.m. for n=4 experiments.
Figure 27. Effect of Ro 25-6981 on NMDA-dependent LTP induction.

Plot showing that the application of 3μM Ro 25-6981 during the HFS-LTP induction protocol was unable to prevent the induction of a significant LTP (170.4±15.7% (p<0.01, n=6) of baseline fEPSP slope. Each point shows the mean±s.e.m. for n=6 experiments.
Figure 28. Role of NR2A and NR2B subunits in long-term potentiation.

Histogram showing LTP induction by high frequency stimulation (HFS, three stimulation trains of 100 pulses at 100 Hz, ten minutes apart). Bath application of either 50μM 2-AP5 or 0.1μM PEAQX prevented the induction of LTP, whereas 3μM Ro 25-6981 did not, reducing LTP to 170.4±15.7% (p<0.01, n=6) of baseline fEPSP slope. This was not significantly less than LTP in the absence of drugs (p=0.2526, n=6), but was significantly greater than the original fEPSP baseline slope (**p<0.01, n=6).
Discussion

The present study has shown that NMDA receptor-dependent LTP induction is critically dependent upon the NR2A subunit-containing receptor activation and not the NR2B subunit in the Schaffer collateral-CA1 synapse. This result is consistent with a recent report that LTP is associated with the rapid surface expression of postsynaptic NR2A subunit-containing NMDA receptors (Grosshans et al., 2002). Additionally, whilst Ro 25-6981 does not block the induction of LTP, the LTP induced was marginally reduced consistent with a recent report (Kohr et al., 2003).

Several reports have now been published investigating the subunit selectivity of LTP and LTD (Sjostrom et al., 2003; Liu et al., 2004; Massey et al., 2004; Mallon et al., 2005). Liu and colleagues reported that at hippocampal CA1 synapses of 3-4 week old rats LTP was blocked by PEAQX. Furthermore, using an LTP protocol pairing Schaffer collateral stimulation with postsynaptic depolarisation under whole-cell recording conditions LTP could be blocked by PEAQX and LTD was induced. The induction of LTD has been reported to be blocked by NR2B antagonists, including Ro 25-6981 and ifenprodil (Sjostrom et al., 2003; Liu et al., 2004; Massey et al., 2004). However the investigation of LTD in this study was prevented because of the difficulty in induction of LTD in adult animals, in agreement with reports by other laboratories (Kemp & Bashir,
Massey and colleagues reported that the induction of LTP and depotentiation in the perirhinal cortex of 7-12 week old rat was blocked by PEAQX. They also found that LTD was difficult to induce but that blockade of glutamate uptake, which led to the activation of postsynaptic, extrasynaptic NR1/NR2B receptors, overcame this obstacle. This allowed the induction of LTD, which could also be prevented by Ro 25-6981, the NR2B antagonist. However, the distribution of NMDA receptor subunits varies widely developmentally and between brain regions and synaptic pathways (Otmakhova et al., 2002; Arrigoni & Greene, 2004). Therefore, different local conditions can regulate the sensitivity and characteristics of synaptic plasticity to different NR2 subunits. It has also been reported that LTP is sensitive to NR2B antagonists in the young Schaffer collateral-CA1 synapse (R. Malenka, personal communication), visual cortex (Yoshimura et al., 2003), amygdala (T. Sigurdsson, personal communication) and the hippocampal commissural-CA3 synapse (Ito et al., 2000). The NR2B subunit, unlike the NR2A subunit-containing receptors or AMPA receptors, seem to be of particular importance in regulating learning and memory and have recently been reported to be differentially distributed in the left-right asymmetry of the brain, which has important implications for higher order functions (Kawakami et al., 2003; Shinohara et al., 2003).
Chapter 5. The NR2A & NR2B subunit-selectivity of homoquinolinic acid

Introduction

The tryptophan metabolite, quinolinic acid (QA), has been recognized for many years to be a weak, endogenous NMDA receptor agonist (Stone & Perkins, 1981; Stone, 1984; 1993). Shortly after its discovery, a study of structure-activity relationships among a series of related compounds revealed that a close analogue, homoquinolinic acid (HQA), had a potent excitatory activity. Indeed, HQA appeared to be between 10 and 100-fold more potent at activating glutamate receptors (Stone, 1984). Autoradiographical and electrophysiological studies have recently been undertaken on native and recombinant receptors to evaluate the subunit-specificity of HQA. It has been reported from electrophysiological studies that HQA is a potent NMDA receptor agonist that has a higher affinity for Xenopus oocytes expressing NR2A and NR2B subunit-containing receptors, compared to NR2C and NR2D subunit-containing receptors (Buller & Monaghan, 1997). A recent study also found that HQA had an approximately 2-fold higher affinity for NR1a/NR2B and NR1a/NR2A/NR2B receptors compared to NR1a/NR2A receptors (EC₅₀ 13.8, 9.04 and 25.2μM, respectively). Yet showed greater intrinsic activity for NR1a/NR2A and NR1a/NR2A/NR2B compared to NR1a/NR2B
transfected receptors (compared to glutamate responses (100%), 148±15, 125±5.6 and 93.3±4%, respectively) (Grimwood et al., 2002). In autoradiographical studies, it has been observed that $^3$H-homoquinolinic acid selectively radiolabels a subpopulation of NMDA receptors in rat brain that is consistent with the distribution of the NR2B subunit and clearly dissimilar to NR2C and NR2D subunit distribution (Buller et al., 1994; De Carvalho et al., 1996; Brown et al., 1998), although there are contradictory reports where HQA was not as highly specific for NR2B-containing receptors (Grimwood et al., 2002). In radioligand binding studies $^3$H-HQA also bound to a novel binding site from which it was not displaced by NMDA or glutamate but was displaced by 2-carboxy-3-carboxymethylquinoline (CCMQ), a HQA derivative (Brown et al., 1998; Grimwood et al., 2002). The identity and function of this proposed site are unknown.

This investigation was designed to establish whether there was any evidence for this unidentified site in the generation of evoked synaptic potentials in the hippocampal slice. Additionally, by using an NR2A/NR2B subunit-containing NMDA receptor agonist (HQA) to investigate subunit-pharmacology in combination with NR2A and NR2B subunit-selective antagonists (PEAQX and Ro 25-6981) this study can consider the role of NR2D subunit-containing receptors on the depression of fEPSP slope and paired-pulse interactions.
Results

HQA and QA

In order to determine the most suitable concentrations of agonists to use concentration-response curves were constructed from which it was clear that HQA and QA gave useful depressions of approximately 50% of the baseline fEPSP slope at 2.5µM and 150µM respectively (figures 29-31, 34-36). These concentrations were selected for further experiments since both increases and decreases of the depression of fEPSP slope could easily be observed. The depression elicited by the application of HQA 2.5µM and QA 150µM both coincided with a significant decrease of paired-pulse inhibition and increase of paired-pulse facilitation at all interstimulus intervals (HQA: 10ms p<0.01, 20ms p<0.001, 50ms p<0.001, n=10; QA: 10ms p<0.05; 20ms p<0.001; 50ms p<0.001; n=7) (figures 32, 33, 37, 38).
Figure 29. The concentration-response curve of HQA.

A plot showing concentration-response curve for homoquinolinic acid-produced depression of the fEPSP slope at 1, 2, 2.5, 3 and 4μM (n = 3, 5, 8, 5 and 4, respectively). The concentration inducing a 50% reduction is 2.4 μM. Each point shows the mean±s.e.m.
Figure 30. Effect of HQA 2.5μM on fEPSP slope.

A plot of fEPSP slope showing that homoquinolinic acid 2.5μM caused a depression of normalised fEPSP slope to approximately 50% (here 45.4% ± 8.12, p < 0.01, n = 8) relative to control responses. Each point shows the mean±s.e.m. for n=8 experiments.
Figure 31. Representative fEPSPs showing effect of 2.5μM HQA.

fEPSPs corresponding to baseline (c) responses and during the depression elicited by HQA 2.5μM (1) in the time course of figure 30.
Figure 32. Effect of 2.5μM HQA on paired-pulse interactions.

Shows the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms during the depression elicited by HQA 2.5μM. The results are presented as the interactions during baseline control responses (c), the effect of homoquinolinic acid 2.5μM (1) and the washout period (2) (10ms p<0.01, 20ms p<0.001, 50ms p<0.001, compared to baseline control responses, n=10), points correspond to the time course of figure 30. The data show a loss of paired-pulse inhibition at 10ms, and increased paired-pulse facilitation at 20 and 50ms caused by homoquinolinic acid. *P < 0.05, **P < 0.01, ***P < 0.001. Each point shows the mean±s.e.m. for n=10 experiments.
Figure 33. Example records of fEPSPs during paired-pulse interactions.

During baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression elicited by HQA 2.5μM: (d) 10ms (e) 20ms and (f) 50ms.
Figure 34. The concentration-response curve of QA.

A plot showing the concentration-response curve of the fEPSP slope to 5-minute applications of QA: 10, 50, 100, 150, 300 and 1000μM (n=4, 3, 6, 8, 3, and 4 respectively). The concentration inducing a 50% reduction is 157.2μM. Each point shows the mean±s.e.m.
Figure 35. Effect of 150μM QA on fEPSP slope.

A plot of fEPSP slope showing that quinolinic acid 150μM caused a depression of normalised fEPSP slope to 55.7%±8.12% (p=0.0017; n=8) relative to control responses. Each point shows the mean±s.e.m. for n=8 experiments.
Figure 36. Representative fEPSPs showing the effect of QA 150μM.

fEPSPs corresponding to baseline (c) responses and during the depression elicited by QA 150μM (1) in the time course of figure 35.
Figure 37. Effect of QA on paired-pulse interactions.

Shows paired-pulse interactions of the fEPSP slope under resting conditions, (c), in the presence of quinolinic acid 150 μM (1), and during washout (2). Quinolinic acid caused a significant decrease of paired-pulse inhibition at 10ms, and a facilitation of the paired-pulse ratio at greater intervals. Points correspond to in the time course of figure 35. *P < 0.05, **P < 0.01, ***P < 0.001 compared to baseline control responses. Each point shows the mean±s.e.m. for n=7 experiments.
Figure 38. Example records of fEPSPs during paired-pulse interactions.

During baseline recording: (a) 10ms (b) 20ms and (c) 50ms and during the depression elicited by QA 150μM: (d) 10ms (e) 20ms and (f) 50ms.
Adenosine is a neuromodulator with an important role in synaptic transmission and neuronal excitability, inhibiting transmission via the activation of presynaptic $A_1$ receptors by decreasing release probability which enhances the paired-pulse interaction (Dunwidde, 1985; Greene, 1991). 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) is a potent and selective adenosine $A_1$ receptor antagonist (Linden, 1991). Since the depolarisation of excitable tissues elicits the release of adenosine, it was important to confirm that the depression of fEPSP slope and the associated changes in paired-pulse interactions were indeed directly attributable to the activation of NMDA receptors and not to the release of endogenous adenosine.

An initial application of HQA $2.5\mu$M caused a depression of baseline fEPSP slope to $13.13\pm3.88\%$ ($p<0.001$, $n=5$). In the presence of DPCPX $20nM$, a concentration that selectively blocks adenosine $A_1$ receptor, the second application of HQA $2.5\mu$M caused a similar depression to $15.20\pm7.04\%$ ($p<0.001$, $n=5$) compared to baseline responses (figure 39). During the initial HQA application the paired-pulse interactions were significantly enhanced at all interstimulus intervals ($p<0.01$, $n=5$ at all intervals) compared to baseline control responses. The co-application of DPCPX during the second application of HQA produced an exactly similar
effect (p<0.01, n=5 at all intervals) compared to baseline control responses (figure 40). This suggests that the presynaptic element of the depression of fEPSP slope is not dependent upon the activation of presynaptic adenosine A₁ receptors.
Figure 39. Effect of DPCPX upon HQA-evoked depression of fEPSP slope.

A plot of fEPSP slope showing the depression of baseline (c) fEPSP slope caused by HQA 2.5μM alone (1), and in the presence of DPCPX 20nM (2). Each point shows the mean±s.e.m. for n=5 experiments.
Figure 40. Effect of HQA and DPCPX upon paired-pulse interactions.

Showing the paired-pulse interactions of fEPSP slope at interstimulus intervals of 10ms, 20ms and 50ms; the results are presented as HQA 2.5μM (1) (p<0.01, n=5 at all intervals) and HQA 2.5μM in the presence of DPCPX 20nM (2) (p<0.01, n=5 at all intervals) both produced significant enhancements of paired-pulse interactions compared with baseline control responses (c). Each point shows the mean±s.e.m. for n=5 experiments.
Inhibition by kynurenic acid, 2-AP5 and dizocilpine

The broad-spectrum EAA antagonist, kynurenic acid (KYA) 100\(\mu\)M, was used at a concentration that selectively blocks NMDA and not AMPA or kainate receptors (Perkins & Stone, 1982; Stone & Burton, 1988). This blocked the depressant effect of both QA and HQA on the evoked fEPSP with no residual response that might indicate a novel binding site (figure 41). The selective and competitive NMDA receptor antagonist 2-AP5 50\(\mu\)M was used, which also blocked the effect of both QA 150\(\mu\)M and HQA 2.5\(\mu\)M but again left no residual effect upon the evoked fEPSP (\(p<0.001\) and \(p<0.001\); respectively, \(n=4\)) (figure 42).

It was thought that the concentration of HQA required to activate the novel binding site might be higher than that required for NMDA receptor activation, but that raising the agonist concentration would simply out-compete the blockade of 2-AP5. Therefore, the selective and non-competitive NMDA receptor antagonist dizocilpine (MK 801) 10\(\mu\)M was used, which produces a voltage and use-dependent non-competitive blockade of the NMDA receptor ion-channel. This blockade allowed the increase by 10-fold the concentration of both QA and HQA to 1.5mM and 25\(\mu\)M respectively so that any novel binding site should be more strongly activated. The blockade of the NMDA receptor was confirmed using NMDA itself at 10\(\mu\)M. However, in the presence of MK 801 these high
concentrations of agonists still did not induce any observable changes in the fEPSP. Unfortunately, it was not possible to perform control responses to these higher concentrations of QA and HQA because, without the presence of the antagonist, they produced a complete and largely irreversible loss of the fEPSP, through excitotoxic neuronal damage (figure 43).
Figure 41. Effect of kynurenic acid on QA and HQA-induced depressions of fEPSP slope.

Representative plot showing the blockade by kynurenic acid 100µM of the depression of fEPSP slope caused by QA 150µM and HQA 2.5µM.
Figure 42. Effect of 2-AP5 on QA and HQA on fEPSP slope.

A plot showing the depression of fEPSP slope during the application of quinolinic acid 150µM and homoquinolinic acid 2.5µM and their subsequent inhibition by 2-AP5 50µM (P < 0.001 and P < 0.001; respectively). Each point shows the mean±s.e.m. for n=4 experiments.
Figure 43. Effect of MK 801 on QA and HQA on fEPSP slope.

A plot showing the inhibition by MK 801 10µM of the depressant effect upon fEPSP slope by NMDA 10µM, QA 1.5mM and HQA 25µM. Each point shows the mean±s.e.m. for n=4 experiments.
Ro 25-6981

As illustrated in figure 44, HQA 2μM (i) and QA 100μM (1) are concentrations that produced small yet discernible depressions of baseline fEPSP slope (c). With the co-perfusion of Ro 25-6981 3μM, the same concentrations of QA (2) and HQA (ii) produced significant depressions of the fEPSP slope to 24.19±15.39% and -1.88±4.93% respectively compared to baseline responses (p<0.001 and p<0.001 respectively n=5). Ro 25-6981 was then allowed to wash out and QA (3) and HQA (iii) were re-applied where a significant depression to 8.98±5.21% and -5.59±4.04% of control respectively was still observed (p<0.001 and p<0.001 respectively n=5) (figure 44). It was noticed that the effect of Ro 25-6981 seemed to develop slowly and did not diminish during the washout phase of the experiment. It was also observed that paired-pulse interactions were significantly enhanced during the enhanced depressions of QA and HQA at all interstimulus intervals after the application of Ro 25-6981 compared to during the initial applications (QA: 10ms p<0.01, 20ms p<0.05, 50ms p<0.01; HQA: 10ms p<0.001, 20ms p<0.001, 50ms p<0.05, n=6; figure 45 and 46).
Figure 44. Effect of Ro 25-6981 with QA and HQA on fEPSP slope.

A plot showing the effect of QA 100μM (1) and homoquinolinic acid 2μM (i) compared to the baseline fEPSP response (c). During the co-perfusion of Ro 25-6981 3μM, the same concentrations of QA (2) and homoquinolinic acid (ii) produced a significant depression to 24.19±15.39% and -1.88±4.93% of the fEPSP slope respectively compared to baseline responses (p<0.001 and p<0.001 respectively). Ro 25-6981 was then washed out for 20 minutes until QA (3) was re-applied and 45 minutes until homoquinolinic acid (iii) was re-applied where a significant depression to 8.98±5.21% and -5.59±4.04% respectively was observed compared to the baseline fEPSP responses (p<0.001 and p<0.001 respectively). Each point shows the mean±s.e.m.
Figure 45. Effect of Ro 25-6981 and QA on paired-pulse interactions.

A plot showing the paired-pulse interactions at interstimulus intervals of 10ms, 20ms and 50ms during baseline recordings (c), the initial application of QA 100μM (1) and the second application of QA 100μM during the co-perfusion with Ro 25-6981 3μM (2) (10ms p<0.01, 20ms p<0.05, 50ms p<0.01, n=6). Points correspond to figure 44. *P < 0.05, **P < 0.01, ***P < 0.001. Each point shows the mean±s.e.m. for n=6 experiments.
Figure 46. Effect of Ro 25-6981 and HQA on paired-pulse interactions.

Showing the paired-pulse interactions at interstimulus intervals of 10ms, 20ms and 50ms during baseline recordings (c), the initial application of HQA 2µM (i) and the second application of HQA 2µM during the co-perfusion of Ro 25-6981 3µM (ii) (10ms p<0.001, 20ms p<0.001, 50ms p<0.05, n=6). Points correspond to figure 44. *P < 0.05, **P < 0.01, ***P < 0.001. Each point shows the mean±s.e.m. for n=6 experiments.
Excitotoxicity

An application of HQA that significantly depressed the fEPSP slope was selected to investigate the effect of Ro 25-6981 on higher concentrations of NMDA receptor agonists. In previous studies of young cultured rat cortical neurones Ro 25-6981 had potentiated small depolarising concentrations of NMDA but blocked larger depolarising concentrations (Fischer et al., 1997). A 5-minute application of HQA 4µM caused a maximal depression of fEPSP slope to 6.63±3.69% (p<0.001, n=4) that reverted to near baseline responses upon washout. Ro 25-6981 3µM was co-perfused with a second application of 4µM HQA which produced a depression of fEPSP slope to -9.27±1.13% (p<0.001, n=4). However, this depression did not fully recover to baseline responses. The fEPSP slope was irreversibly reduced to an average 34.70±0.38% compared to baseline responses (p<0.001, n=4). Maximum evokable amplitudes of fEPSP slope (100%), which were taken as an additional measure of excitotoxic damage, were significantly reduced (p=0.0011, n=4) compared to the baseline maximums recorded and they did not recover during the 70-minute washout phase of the experiment. Attempts to induce LTP at the end of the experiments also failed, again suggesting that irreversible excitotoxic damage had occurred (figure 47 and 48). This specifically ruled out the induction of NMDA-receptor dependent LTD, which was reversible by high frequency stimulation (data not shown).
A 5-minute application of homoquinolinic acid 4μM elicited a reversible depression of baseline fEPSP slope to 6.63±3.69% (p<0.001). Ro 25-6981 3μM was co-perfused and a second 5-minute application of homoquinolinic acid 4μM produced a depression of fEPSP slope to -9.27±1.13% (p<0.001). However, the fEPSP slope did not fully recover but was significantly and irreversibly reduced to an average 34.70±0.38% compared to the initial baseline (p<0.001). Each point shows the mean±s.e.m. for n=4 experiments.
Figure 48. Representative fEPSP recordings showing excitotoxicity.
During baseline recording (c) and during the irrecoverable depression (1) corresponding to figure 47.
NR2A antagonists

HQA is reported to be a selective agonist for NR2A and NR2B subunit-containing NMDA receptors. Using Ro 25-6981, an NR2B antagonist, to produce the potentiation of the depression of fEPSP slope by low concentrations of HQA suggests that the remaining HQA-dependent effect could be modulated by the remaining NR2A component of HQA activity. In order to test this theory several candidate NR2A selective antagonists including zinc, CCMQ and PEAQX were investigated (Paoletti et al., 1997; Brown et al., 1998; Auberson et al., 2002; Grimwood et al., 2002).

CCMQ

Previous autoradiographical studies found that 2-carboxy-3-carboxymethylquinoline (CCMQ), a derivative of HQA, displaces bound $[^3]$H-HQA from its NMDA-insensitive novel binding site at a concentration of 1mM, leaving behind a binding pattern which closely resembles the distribution of NR2B subunits in the rat brain (Brown et al., 1998; Grimwood et al., 2002). These results and the structural homology with HQA suggest that CCMQ may be able to displace HQA from the NR2A subunit-containing NMDA receptors thereby leaving only the NR2B population, acting as an NR2A-selective ligand.
The effect of CCMQ 0.5mM upon the HQA-induced depression of fEPSP slope was investigated first. As shown in figure 49 two initial applications of HQA 2.5μM produced two similar depressions of fEPSP slope. The third application of HQA was co-applied with CCMQ but this had no effect on an identical depression of fEPSP slope. This suggests that CCMQ was not acting as an antagonist of the NR2A site activated by HQA. This study next investigated if CCMQ was an NR2A agonist and used Ro 25-6981 to block the NR2B subunit in order to help reveal any NR2A effect. As shown in figure 50 the perfusion of Ro 25-6981 alone had no effect upon baseline fEPSP slope but CCMQ elicited a small potentiation of the fEPSP slope to 108.65±1.71% (p=0.0058, n=3). Finally, the effect of CCMQ upon the potentiation of HQA 2μM caused by Ro 25-6981 3μM was investigated in order to address the subunit specificity of this potentiation. As shown in figure 51 the application of HQA 2μM produced little response but the application of Ro 25-6981 potentiated this to a significant depression to 14.72±10.49% (p<0.001, n=3) of baseline fEPSP slope, which was not inhibited by CCMQ. The co-perfusion of CCMQ resulted again in the same small but significant increase in fEPSP slope to 124.25±2.75% (p<0.05, n=3). A third application of HQA 20 minutes after the end of the perfusion of Ro 25-6981 also produced a large depression to 7.18±3.24% (p<0.001, n=3) of fEPSP slope.
Figure 49. Effect of HQA 2.5μM and CCMQ 0.5mM on fEPSP slope.

A plot showing the effect of repeated 5-minute perfusions of homoquinolinic acid 2.5μM, that caused three consecutive depressions of the fEPSP slope. The co-perfusion of CCMQ 0.5mM with the third perfusion of homoquinolinic acid had no significant effect upon the peak magnitude or duration of the response. Each point shows the mean±s.e.m. for n=3 experiments.
Figure 50. Effect of CCMQ and Ro 25-6981 on fEPSP slope.

A plot showing the effect of 3μM Ro 25-6981 and the subsequent co-perfusion of 0.5mM CCMQ which produced a small potentiation of the fEPSP slope to 108.65±1.71% (p=0.0058). Each point shows the mean±s.e.m. for n=3 experiments.
Figure 51. Effect of HQA 2μM, Ro 25-6981 and CCMQ 0.5mM on fEPSP slope.

Plot showing that the first application of HQA 2μM produced little response but the co-application of Ro 25-6981 potentiated a second HQA-application producing a significant decrease to 14.72±10.49% (p<0.001) of baseline fEPSP slope. The co-perfusion of CCMQ again resulted in the same small but significant increase in fEPSP slope to 124.25±2.75% (p<0.05). A third application of HQA 20 minutes after the end of the perfusion of Ro 25-6981 also produced a large depression to 7.18±3.24% (p<0.001) of fEPSP slope. Each point shows the mean±s.e.m. for n=3 experiments.
Zinc ions (Zn$^{2+}$) have been reported to produce a selective and non-competitive inhibition of the NR2A subunit-containing recombinant NMDA receptors, producing a voltage-independent inhibition at nanomolar concentrations (Paoletti et al., 1997). As shown in figure 52 an initial application of HQA 2.5μM caused a significant depression of fEPSP slope to $0.7\pm0.57\%$ (p<0.001, n=4), a second application of HQA with Zn$^{2+}$ 100nM also produced an identical depression of fEPSP slope to $2.6\pm2.2\%$ (p<0.01, n=4). Thus, 100nM Zn$^{2+}$ was unable to prevent the depression of fEPSP slope.
Figure 52. Effect of HQA 2.5μM and Zn²⁺ 100nM on fEPSP slope.

A plot showing that the first application of HQA 2.5μM caused a significant depression of baseline fEPSP slope to 0.7±0.57% (p<0.001). A second application of 2.5μM HQA with 100nM Zn²⁺ again produced a similar depression of fEPSP slope to 2.6±2.2% (p<0.01). Each point shows the mean±s.e.m. for n=4 experiments.
Having been unsuccessful with CCMQ and zinc in attempts to antagonise the NR2A subunit, PEAQX, a novel NR2A antagonist was obtained as a gift from Dr Yves Auberson of Novartis Pharmaceuticals. This compound has 126-fold selectivity for the NMDA NR1A/NR2A over the NR1A/NR2B receptors expressed in Xenopus oocytes (IC\textsubscript{50} = 14nM and 1800nM respectively)(Auberson et al., 2002). This investigation was started by constructing a concentration-response curve for the inhibitory effect of PEAQX upon the depression of fEPSP slope elicited by HQA 3µM. This was achieved by applying 3µM HQA, which produced a large depression of the fEPSP slope, and then PEAQX was co-applied at varying concentrations. Subsequent co-applications of the same concentration of HQA were made and the degree of antagonism produced was observed (figures 53 and 54). From the concentration-response curve it was determined that using a concentration of 0.1µM provided the most selective and complete antagonism of the NR2A subunit-containing NMDA response. This concentration-response curve is very similar to that found for the effect of PEAQX on NMDA-induced currents in Xenopus oocytes expressing human NR1A/2A subunits (Appendix 2).

This study also investigated paired-pulse interactions during the interaction of HQA and PEAQX. It was found that during the initial depression of
fEPSP slope caused by HQA 3μM the paired-pulse ratios were increased at all interstimulus intervals (10ms p<0.001, 20ms p<0.01, 50ms p<0.001; n=6) but that during the subsequent co-application of PEAQX 0.1μM and HQA 3μM they remained similar to baseline responses (figure 55).
Figure 53. Effect of HQA and PEAQX on fEPSP slope.

A plot showing that the control application of HQA 3μM (1) produced a significant depression of baseline fEPSP slope (c) to 20.44±0.59% (p=0.0019). The depression of fEPSP slope expected by the second application of HQA 3μM was prevented by the co-application of PEAQX 0.1μM (2). Each point shows the mean±s.e.m. for n=6 experiments.
Figure 54. The concentration-response curve of PEAQX on HQA-induced depression of fEPSP slope.

A plot showing the concentration-response curve of the inhibitory action of PEAQX at concentrations of 1nM, 5nM, 10nM, 50nM, 100nM and 500nM (n=3 at all intervals, except n=6 for 100nM PEAQX) upon HQA 3μM, which normally caused a significant depression of fEPSP slope. A concentration of 9.23nM is predicted to produce a 50% inhibition of the depression elicited by HQA 3μM. Each point shows the mean±s.e.m.
Figure 55. Effect of HQA and PEAQX on paired-pulse interactions.

A plot showing the paired-pulse interactions during baseline responses (c), which were significantly enhanced at interstimulus intervals of 10ms, 20ms and 50ms during the first application of HQA 3μM (1) and returned to control levels during the blockade of the second application of HQA 3μM by PEAQX 0.1μM (2). Points correspond to the time-course in figure 53. Each point shows the mean±s.e.m. for n=6 experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
PEAQX's inhibition of the Ro 25-6981/HQA potentiation.

However, of greater interest was the finding that the potentiated depression of fEPSP slope obtained by HQA 2µM with Ro 25-6981 3µM was also completely blocked by PEAQX 0.1µM. As shown in figure 56, a concentration of 2µM HQA (1) initially produced a small and insignificant depression of the baseline fEPSP slope (c) but following the perfusion of Ro 25-6981 3µM, a second application of HQA 2µM (2) produced a significant depression of fEPSP slope to 37.33±4.43% (p=0.0261, n=4). The co-perfusion of PEAQX 0.1µM prevented the depression of a third application of HQA 2µM (3).

It was also observed that paired-pulse interactions during the first application of HQA 2µM (1) were not significantly different from baseline responses (c). However, after the application of Ro 25-6981 3µM, the second application of HQA 2µM (2) caused a significant enhancement of paired-pulse ratios (p<0.001, n=4 at all interstimulus intervals). During the inhibition by PEAQX 0.1µM, the third application of HQA 2µM (3) saw paired-pulse interactions remain similar to baseline responses (figure 57).
Figure 56. Effect of HQA, Ro 25-6981 and PEAQX on fEPSP slope.

A plot showing the effect of a low concentration of homoquinolinic acid 2μM (1) that initially produced a small, insignificant depression of the baseline fEPSP slope (c). Following the perfusion of Ro 25-6981 3μM, a second application of homoquinolinic acid 2μM (2) produced a significant depression of fEPSP slope to 37.33±4.43% (p=0.0261). The co-perfusion of PEAQX 0.1μM, an NR2A selective antagonist, inhibited the depression of the third application of homoquinolinic acid 2μM (3). Each point shows the mean±s.e.m. for n=4 experiments.
Figure 57. Effect of HQA, Ro 25-6981 and PEAQX on paired-pulse interactions.

This plot shows the paired-pulse interactions recorded during the co-perfusions of HQA 2µM, Ro 25-6981 3µM and PEAQX 0.1µM. Points correspond to the time course of figure 56. Paired-pulse interactions during the first application of homoquinolinic acid 2µM(1) were not significantly different from baseline responses (c). However, after the application of Ro 25-6981 3µM the second application of homoquinolinic acid 2µM (2) caused an enhancement of the paired-pulse interactions at 10ms (p<0.001), 20ms (p<0.001) and 50ms (p<0.001) compared to baseline interactions. During the NR2A inhibition of PEAQX the third application of homoquinolinic acid 2µM (3) saw the paired-pulse interactions return to baseline responses (n=4). Each point shows the mean±s.e.m. for n=4 experiments. *P < 0.05, **P < 0.01, ***P < 0.001. Points correspond to the time course in figure 56.
Protein synthesis

In view of the latency of onset, the long-lasting effect of Ro 25-6981 and the involvement of a protein phosphatase signal transduction step the question arose of whether any new protein synthesis was required and induced. To investigate this possibility anisomycin 30μM was used, which has previously been shown to block the maintenance of LTP and LTD at this concentration and over this timescale (Grollman, 1967; Otani et al., 1989; Manahan Vaughan et al., 2000; Karachot et al., 2001). An initial application of HQA 2μM caused a small depression to 82.68±3.16% of baseline fEPSP slope. Anisomycin 30μM and the subsequent co-perfusion of Ro 25-6981 3μM had no significant effect on the baseline fEPSP slope. A second application of HQA 2μM produced a large and significant depression of fEPSP slope to 0.91±3.0% (p=0.0152, n=4) compared to the initial application of HQA 2μM. A similar profound depression of fEPSP slope to 6.05±4.86% (p=0.0128, n=4), again in comparison to the initial application of HQA 2μM, was obtained with a subsequent application of HQA 2μM (figure 58). This suggests that new protein synthesis is not required for the potentiation by Ro 25-6981.
Figure 58. Effect of HQA, Ro 25-6981 and anisomycin upon fEPSP slope.

Plot showing that an initial application of homoquinolinic acid 2μM caused a small depression of baseline fEPSP slope, the perfusion of anisomycin 30μM and subsequent co-perfusion of Ro 25-6981 3μM had no significant effect on the baseline fEPSP slope. The second application of homoquinolinic acid 2μM produced a depression to 0.91±3.04% (p=0.0152, n=4) compared to the trough of the initial application of homoquinolinic acid 2μM. After washing out for 20 minutes a further application of homoquinolinic acid 2μM caused a similar depression of fEPSP slope to 6.05±4.86% (p=0.0128, n=4) again in comparison to the trough depression of the initial application of homoquinolinic acid 2μM. Each point shows the mean±s.e.m. for n=4 experiments.
AMPA and kainate receptors

The earlier paired-pulse data suggested that there might be a presynaptic component during the NMDA, HQA and QA evoked depressions of fEPSP slope. AMPA and kainate receptors are the main glutamatergic receptors responsible for basal synaptic transmission and the generation of currents underlying the fEPSP. AMPA and kainate were used at 100nM and 25nM respectively, concentrations that produced small depressions of the fEPSP slope. In both cases, the application of Ro 25-6981 3μM had no effect on the responses, confirming that this effect is specific to NMDA receptor activation (figures 59 and 60). This is consistent with the absence of effect of Ro 25-6981 alone upon baseline responses.
Figure 59. Effect of Ro 25-6981 and AMPA on fEPSP slope.

Plot showing the effect of 100nM AMPA applied before and after the perfusion of 3μM Ro 25-6981, the response to AMPA was unchanged. Each point shows the mean±s.e.m. for n=3 experiments.
Figure 60. Effect of Ro 25-6981 and KA on fEPSP slope

Plot showing the effect of 25nM kainate applied before and after the perfusion of 3μM Ro 25-6981, the response to kainate was unchanged. Each point shows the mean±s.e.m. for n=3 experiments.
Discussion

In order to expose a response to the activation by homoquinolinic acid of the uncharacterised binding site reported by others the non-selective EAA antagonist kynurenic acid, the competitive NMDA receptor antagonist 2-AP5 and the non-competitive antagonist dizocilpine (MK-801) were used. 2-AP5, as with some competitive antagonists (straight chain, piperidine and piperazine structures) displays the subunit potency NR2A > NR2B > NR2D > NR2C (Laurie & Seeburg, 1994). However, neither kynurenic acid, 2-AP5, dizocilpine nor PEAQX revealed a novel action of homoquinolinic acid on the fEPSP slope. Therefore, the novel binding site for homoquinolinic acid does not affect acutely recorded synaptic potentials in the hippocampus and homoquinolinic acid appears to be a selective agonist at NMDA receptors that include the NR2A subunit.

Particularly when studied on fEPSPs, the paired-pulse paradigm represents a valuable means of assessing the actions of compounds on presynaptic terminals. The paired-pulse inhibition obtained at interpulse intervals of 10ms results from the depletion of transmitter from presynaptic stores (Burke & Hablitz, 1994; Wilcox & Dichter, 1994; Hashimoto & Kano, 1998), and is reduced by agents or procedures which decrease transmitter release. Paired-pulse facilitation, observed at longer interpulse intervals, is due to the residual intraterminal Ca$^{2+}$ which increases transmitter release.
(Hess et al., 1987; Wu & Saggau, 1994; Debanne et al., 1996; Kleschevnikov et al., 1997). The modification of paired-pulse interactions by homoquinolinic acid and quinolinic acid suggests that at least part of its ability to depress the fEPSP slope is presynaptic in origin. Indeed, there is ample evidence corroborating the existence of presynaptic NMDA receptors in several regions of the brain (Berretta & Jones, 1996; Hamada et al., 1998; Glitsch & Marty, 1999; Woodhall et al., 2001), including the hippocampus (Breukel et al., 1998). Alternatively, a retrograde signalling molecule released postsynaptically in response to depolarisation by homoquinolinic acid may mediate this effect indirectly or other postsynaptic effects may confound the changes in PPD and PPF (Foster & Richardson, 1997). The possibility that adenosine released in response to neuronal depolarisation could act as a retrograde inhibitor was investigated using the selective A1 receptor antagonist DPCPX. However, DPCPX had no effect upon the magnitude of the depression caused by homoquinolinic acid or paired-pulse interactions suggesting that the presynaptic activity of homoquinolinic acid is not dependent upon the activation of presynaptic adenosine A1 receptors.

As previously seen with NMDA, Ro 25-6981 potentiated the responses to HQA and QA on fEPSP slope and altered paired-pulse interactions. One possibility for the potentiating effect of Ro 25-6981 is that it causes an increase in the release probability of glutamate from the presynaptic
boutons. This is unlikely as there is no effect of Ro 25-6981 on baseline fEPSP or on comparable responses to AMPA and kainate. Another possibility is that Ro 25-6981 increases the local release of an endogenous NMDA agonist such as glycine, homocysteate, cysteine sulphinate or aspartate (Patneau & Mayer, 1990; Davanger et al., 1994). Whilst glycine has a high affinity and is thought to saturate NR1/NR2B receptors, it has a lower affinity for NR1/NR2A receptors, which under resting concentrations of glycine could be unsaturated and may be potentiated with rising glycine concentrations.

CCMQ was not able to block the depression of fEPSP slope at a similar concentration at which it displaced the binding of HQA in binding studies. Furthermore, the Ro 25-6981-enhanced response to HQA was similarly not blocked. This suggests that CCMQ is not acting as an antagonist in its ability to displace HQA from NR2A subunits in the previous studies. Considering the slight, yet significant, increases in fEPSP slope elicited by CCMQ under the influence of Ro 25-6981 it is possible that CCMQ is a weak NR2A agonist. This will best be confirmed using recombinant NR1/NR2A receptors expressed in Xenopus oocytes. Once this has been achieved, a study of structure-activity relationships could lead to more potent and selective NR2A agonists.

The absence of effect of zinc as an NR2A antagonist may be due to the experimental conditions where the level of NR2A inhibition is already
saturated by the concentrations of zinc contaminating the ACSF. Also there may be physiological mechanisms that regulate zinc inhibition still active in the intact slice preparation, such as tyrosine kinase src phosphorylation and chelation of zinc by thiol-reducing agents (Paoletti et al., 1997; Arden et al., 1998; Zheng et al., 1998). In addition, differences between native heteromeric receptors and the recombinant receptors may weaken the effectiveness of zinc. Zinc is concentrated by certain neurones into synaptic vesicles and can be released in a Ca$^{2+}$-dependent manner at some synapses including the mossy fibre-CA3 pyramidal synapse (Dingledine et al., 1999).

The effect of the NR2A antagonist PEAQX demonstrates that the NR2A subunit-containing NMDA receptors gate the currents involved in the depression of fEPSP slope and the associated paired pulse interactions. The enhancements of paired-pulse interactions are associated both with HQA alone and under the influence of Ro 25-6981, where the NR2B subunit is in both an activated and inhibited state. This suggests that the NR2B subunit cannot independently alter the presynaptic element suggested by paired-pulse results although it may be able to do so indirectly by its modulation of NR2A subunits, which alone seems to modulate directly the enhancement of paired-pulse interactions and the depression of the fEPSP slope.
Chapter 6. General Discussion

Negative coupling of NR2B to NR2A subunit-containing receptors

This study has suggested that acutely activated, tonically activated or constitutively active NR2B subunits maintain an inhibitory restraint upon the activation of the NR2A subunit-containing NMDA receptors by way of a protein phosphatase 2B (calcineurin) transduction step. The NR2A subunit-containing receptor seems to be the principal receptor involved in postsynaptic depolarisation and presynaptic alteration of release probability. The NR2B subunit has no observable function other than to gate the activity of the NR2A subunit-containing NMDA receptors. A recent report investigating the properties of NR1/NR2B/NR2D receptors expressed in newborn rat hippocampal granule cells has also observed evidence of negative coupling (Pina Crespo & Gibb, 2002). Additionally, NR3A subunits have been shown to negatively modulate NMDA receptors (Das et al., 1998). Although the effect of Ro 25-6981 is dependent on the presence of an NMDA receptor agonist to observe its effect, it is a persistent change in the weight of the synapse over a timescale reported to be associated with a potentiation of NMDA (Watt et al., 2004). Furthermore, it may involve co-incident pre- and postsynaptic activity suggestive of a Hebbian process. The NMDA receptor has a central role in
the induction of LTP and LTD, the negative coupling demonstrated in this study has significant implications for determining both the threshold for LTP and LTD induction and perhaps the magnitude of plasticity induced. The cellular mechanism by which this enhancement is produced is not entirely understood. Such an enhancement has very exciting implications for our understanding of NMDA receptor physiology and pharmacology.

**Multimeric NMDA receptor composition**

Nicotinic acetylcholine receptors are pentamers, voltage-gated potassium channels are tetramers and gap junction proteins mediating coupling at electrical synapses are hexameric (or hexamonic). Glutamate receptors were often supposed to be pentamers because they, like the acetylcholine receptor are ligand gated. Equal evidence exists for both a tetrameric or a pentameric structure from receptor subunit studies (Behe et al., 1995; Ferrer Montiel & Montal, 1996; Premkumar & Auerbach, 1997; Laube et al., 1998; Mano & Teichberg, 1998; Rosenmund et al., 1998). However, these studies are designed to identify the number of functional binding sites in a receptor but are based upon the assumption that each binding site in a receptor operates independently of other subunits. This study has suggested that there is a negative coupling between the NR2A and the NR2B receptor subunits and it has been reported by others of a negative coupling in receptors containing NR3A and NR2A subunits (Ciabarra et al., 1995; Das et al., 1998) and NR2B and NR2D subunits (Pina Crespo &
Gibb, 2002). This observation suggests that the analysis of dose-response curves (Laube et al., 1998) and patterns of main and sub-conductance states (Behe et al., 1995; Premkumar & Auerbach, 1997) may be more complicated and the heteromeric structure of the native NMDA receptor may have been miscalculated.

**Presynaptic NMDA receptors**

Demonstrating the role of presynaptic receptors definitively ideally requires the fulfilment of five criteria (McGehee & Role, 1996; MacDermott et al., 1999):

1. Evidence for the presence of presynaptic receptors.

2. Activation of these receptors exogenously produces the presynaptic effect that alters release probability (that mimics physiological control).

3. Presence of an endogenous ligand at the synapse that can be released under physiological conditions for activation of the presynaptic receptor.

4. Antagonists that block the presynaptic ionotropic receptors block synaptic plasticity.
5. Activation of the presynaptic receptor leads to the observed change in release through a characterised mechanism.

In answer to these criteria:

1. AMPA, kainate and metabotropic glutamate receptors have all been established as presynaptic autoreceptors in the hippocampus (Vazquez et al., 1994; Chittajallu et al., 1996; Alici et al., 1997), modulating excitatory transmission by altering glutamate release probability. NMDA receptors have been amply demonstrated on the postsynaptic membrane (Petralia et al., 1994; Racca et al., 2000) and there is accumulating evidence demonstrating a presynaptic locus. Presynaptic NMDA autoreceptors have been reported in the cerebellum (Glitsch & Marty, 1999), spinal cord (Liu et al., 1997), hippocampus (Breukel et al., 1998), suprachiasmatic nucleus (Hamada et al., 1998) and entorhinal cortex (Berretta & Jones, 1996). Furthermore, presynaptic NMDA receptors have been reported to be NR2B subunit-containing and tonically activated, causing an increase in glutamate release probability, dependent upon Ca\(^{2+}\) influx, in the entorhinal cortex (Woodhall et al., 2001). Additionally, immunohistochemical studies have found evidence for presynaptic NMDA receptors (Liu et al., 1994; Paquet & Smith, 2000).
2. This report has suggested, through the changes observed in paired-pulse interactions, that NMDA receptor activation by HQA, QA and NMDA generated an effect with a presynaptic element. However, whilst PPF and PPD are generally accepted to be presynaptic phenomenon, it does not follow that postsynaptic effects cannot influence their appearance in field recordings (Foster & Richardson, 1997).

3. The endogenous ligand at these synapses is glutamate, which could act under physiological conditions to activate a presynaptic receptor. Furthermore, this study has demonstrated this effect by using another endogenous ligand quinolinic acid.

4. This investigation has demonstrated that both 2-AP5 and PEAQX can block NMDA-receptor dependent LTP. Furthermore, it has been reported recently that Ro 25-6981 is able to inhibit LTD (Sjostrom et al., 2003; Liu et al., 2004; Massey et al., 2004)

5. The inhibition of this effect by cyclosporin A, which modulates protein phosphatase 2B (calcineurin) activity, demonstrates a distinct and well-characterised signal transduction pathway.

The lack of sufficiently compelling evidence on point 2 in this study means that there is not full qualification for a presynaptic NMDA receptor.
Therefore, the possible involvement of a postsynaptic receptor with a retrograde signalling mechanism must be considered. In NMDA receptor dependent synaptic plasticity, postsynaptically induced processes such as LTD and LTP are still able to trigger presynaptic changes through retrograde messengers such as nitric oxide activating PKG (Reyes Harde et al., 1999) and endocannabinoids acting upon CB1 receptors (Chevaleyre & Castillo, 2003; Sjostrom et al., 2003) or release from other sources such as glia (Parpura et al., 1994).

Whilst changes in paired-pulse interactions are widely regarded to be due to presynaptic alterations in release probability; when studied on fEPSPs and especially where there are changes in the size of the postsynaptic potential in response to the test agents many factors affect them which can affect their interpretation. Including, changes in presynaptic release probability (Andreasen & Hablitz, 1994), activity of presynaptic autoreceptors on inhibitory or excitatory terminals (Davies et al., 1990; Stanford et al., 1995), modulation of postsynaptic conductances and the presence of GABA-ergic inhibition following the fEPSP and the depression cause by NMDA agonist action (Duguid & Smart, 2004).

Also, considering the intracellular data showing a purely postsynaptic manifestation of these effects a further study of the contribution of different NMDA subunits is required on clear presynaptic phenomenon such as spontaneous miniature EPSPs or by concurrently measuring by patch-
clamp whole cell recording corresponding presynaptic and postsynaptic neurones.

It is clear that NR2A and NR2B subunit-containing NMDA receptors are found postsynaptically, as NR1/NR2A, NR1/NR2B or NR1A/NR2A/NR2B heteromers, at the synaptic site whilst NR1/NR2B receptors are found exclusively at the extrasynaptic site (Li et al., 1998; Stocca & Vicini, 1998; Tovar & Westbrook, 1999). A tonically activated state of the agonist-preferring NR2B subunit-containing receptor could be achieved by being located near to the release site of glycine and glutamate on the presynaptic bouton. However, the NR2B subunit in both its activated and inhibited state is associated with enhancements in paired-pulse inhibition whereas, only the blockade of the NR2A subunit prevents the enhancement of paired-pulse interactions. Alternatively, the NR2B subunit may be in a constitutively active state that is independent of agonist binding as has been observed with other receptors (Scheer & Cotecchia, 1997; Behan & Chalmers, 2001; Richard et al., 2001). Interestingly NR2B subunit-containing receptors have a higher glutamate and glycine affinity, are less sensitive to tonic proton inhibition, and are insensitive to desensitisation, which suggests that they would be more sensitive detectors of glutamate than NR2A subunit-containing receptors, especially at extrasynaptic sites. Applications of HQA, QA and NMDA by themselves and under the influence of NR2B antagonism are associated with depressions of fEPSP.
slope and increases in paired-pulse ratios. These are both blocked by PEAQX, the NR2A antagonist, suggesting that the presynaptic element may be NR2A subunit-mediated. It is difficult from the current study to make any definitive resolution of the receptor populations found pre- and postsynaptically or the subunit makeup of such receptors.

It may be possible to investigate these further using single channel analysis of outside-out patches from these tissues. Using selective antagonists, it can be determined whether the negative coupling of NR2B upon NR2A is between subunits in a single heterotrimeric NR1/NR2A/NR2B receptor (rather than between discrete heterodimeric NR1/NR2A and NR1A/NR2B receptors). This may also address the location of these subunits at pre- or postsynaptic sites. There is evidence for the co-localisation of NR2A and NR2B subunits in the hippocampus, and specifically within the CA1, CA2 and CA3 regions (Fritschy et al., 1998).

**Excitotoxicity**

This report has demonstrated that, far from being neuroprotective, Ro 25-6981 actually precipitates an excitotoxic insult from otherwise benign, depolarising concentrations of NMDA and HQA. This observation is contradictory to previous reports of NR2B antagonists being neuroprotective in excitotoxic studies (Gotti et al., 1988; Fischer et al., 1997; Lynch et al., 2001; Gill et al., 2002) but some reports have
corroborated this study (Zou & Crews, 2003). The age of tissue used in investigations could account for the reason for the difference in these results. In previous studies immature tissues were used whereas this study has used adult tissues in a well-proven experimental model of excitotoxicity (Schurr et al., 1995). As mentioned in the introduction, the expression levels and distribution of NMDA subunits change dramatically during development, especially in respect of the NR2A and the NR2B subunits. This developmental change is responsible for the alteration in susceptibility of tissues to excitotoxicity and also to the induction of synaptic plasticity (Dudek & Bear, 1993; Liu et al., 1996). The use of immature tissues to investigate excitotoxicity is dictated to some extent by methodological constraints. The rationale for using these tissues in the investigation of stroke, which is an almost exclusive threat to the adult population, is difficult to justify, especially here, considering the disparity between these results. Additionally, the study of tissue from different areas of the brain and even different regions of the hippocampus will dictate the influence of NMDA receptors and the expression of different types and quantity of NR2 subunits. A recent study of cultured hippocampal neurones reported that the selective activation of synaptic NR2A subunit-containing NMDA receptors is associated with the potent activation of cAMP response element binding protein (CREB) and subsequent brain-derived neurotrophic factor (BDNF) gene expression. This leads to an anti-apoptotic cell-signalling pathway. However, the activation of NR2B
sensitive extrasynaptic NMDA receptors by bath application of NMDA agonists or exposure to hypoxic/ischaemic conditions, which cause the reversal of glutamate transporters (Rossi et al., 2000), is associated with the Ca	extsuperscript{2+}-dependent transient activation of CREB. This leads to a CREB shut-off signal of the synaptic pro-survival pathway and leads to cell death (Hardingham et al., 2002). It can be concluded that excessive overactivation of the NR2A-subunit associated channel could be directly responsible for the large Ca	extsuperscript{2+} influx that triggers excitotoxicity. The NR2B subunit acting to gate the activity of the NR2A subunit-containing receptor and when it is inhibited this restraint is lost.

An obvious question arises as to whether there a correlation between decreased levels of NR2B subunit expression, mRNA levels and genetic expression in adult patients susceptible to stroke or in geriatric patients who have an age-related susceptibility to stroke and in other relevant disease states. Indeed, the NR2B subunit is already implicated in an impressive number of age related diseases and developmentally, the mRNA and the protein expression of the NR2B subunit decreases significantly between the ages of 3 and 30 months in mice (Magnusson, 2000; Magnusson et al., 2002) which is similar to the decrease in the human hippocampus (Law et al., 2003). It is possible to further develop this study by investigating the effect of selective subunit NMDA antagonists in hypoxia, ischaemia and seizure disorder, which are known to have NMDA receptor components.
The potential for the development of drugs to modulate this NR2B-NR2A interaction is very exciting.

**Phosphatase hypothesis**

This report has suggested that NR2B subunits maintain an inhibitory control over NR2A subunit-containing receptors. It could achieve this by being acutely activated, tonically activated or remaining constitutively active physiologically, maintaining the phosphorylation state of an unidentified protein. The superfusion of Ro 25-6981 blocks the NR2B subunit leading to the calcineurin-mediated dephosphorylation of an unknown protein residue resulting in the potentiation of NR2A subunit-containing NMDA receptor responses. NR2 subunits are known to be highly phosphorylated under basal conditions whilst NR1 subunits are very weakly phosphorylated (Lau & Huganir, 1995; Hall & Soderling, 1997; Smart, 1997). Therefore, the dephosphorylation step described here may be acting directly upon either the NR2A or the NR2B subunit to produce the enhancement of responses to NMDA and HQA. However, it may also be acting on the NR1 subunit or associated proteins such as PSD-95 and SAP 102 or an unrelated and possibly novel site. Interestingly, there is strong evidence for a tyrosine residue that is phosphorylated and leads to potentiation of the NMDA receptor and is found on the NR2A or NR2B subunit-containing receptor, as seen with insulin potentiation and LTP (Chen & Leonard, 1996; Kohr & Seeburg, 1996; Rosenblum et al., 1996; 187
Smart, 1997; Zheng et al., 1998; Christie et al., 1999). The absence of effect of okadaic acid, a potent inhibitor of the serine/threonine protein phosphatases types 1 and 2A, rules out these serine residues being involved. The next step is to identify if there are changes in the levels of phosphorylation of these subunits. This can be achieved by a combination of immunoprecipitation using NR1, NR2A and NR2B subunit specific antibodies to these proteins, then immunoblotting with anti-phosphotyrosine and anti-phosphoserine specific antibodies and estimating changes in levels of phosphorylation compared to controls with a chemiluminescent secondary antibody. There are many studies that correlate increases in phosphorylation with increases in receptor activity and that dephosphorylation is associates with reductions in receptor activity (Smart, 1997). However, it was found in this study that the activity of calcineurin, which de-phosphorylates is associated with the potentiation of responses to NMDA. This is blocked by Cyclosporin A, a phosphatase inhibitor. Many of the previous studies were carried out using immature tissue as with previous excitotoxicity reports as mentioned previously. In addition to the developmental switch of subunit expression (Tovar & Westbrook, 1999) the basal levels of phosphorylation of the adult NMDA receptor do not reach maturity until 3-4 weeks post-natally (Cudmore & Gurd, 1991).
Synaptic plasticity

The entry of calcium into the neurone contributes \( \sim 16\% \) of the NMDA receptor current but the subsequent activation of \( \text{Ca}^{2+} \)-dependent enzymes and signal transduction pathways induce potent effects (Schneggenburger et al., 1993). Both LTP and LTD can be induced by NMDA receptor activation, resulting in an influx of \( \text{Ca}^{2+} \) that triggers plasticity. The relative level, kinetics and location of the \( \text{Ca}^{2+} \) influx determining whether LTP or LTD is induced; a large, fast, synaptic influx inducing LTP and a relatively modest, prolonged, extrasynaptic influx inducing LTD (Lisman, 1989; Artola & Singer, 1993; Neveu & Zucker, 1996; Yang et al., 1999; Cho et al., 2001; Liu et al., 2004; Massey et al., 2004). The receptor kinetics of the NR2B subunit-containing receptor is relatively slower than the NR2A subunit-containing receptor resulting in calcium influxes that are consistent with LTD and LTP induction respectively (figure 61). This suggests that there is a role of NR2B in LTD that requires an NMDA-mediated influx of \( \text{Ca}^{2+} \) consistent with the NR2B subunit-containing NMDA receptor kinetics. Indeed, it has recently been reported that neocortical LTD is inhibited by NR2B antagonists affecting presynaptic receptors (Sjostrom et al., 2003), that LTD in the perirhinal cortex is inhibited by the NR2B antagonist Ro 25-6981 but not by the NR2A antagonist PEAQX (Massey, 2003; Massey et al., 2004) and that LTD in the hippocampus is NR2B dependent (Liu et al., 2004). The NR2A
subunit-containing receptor has kinetics consistent with those required for a large and rapid rise in intracellular calcium concentrations leading to LTP induction. During LFS protocols that induce LTD the NR1/NR2B receptors make a larger contribution to the total charge transfer and Ca\(^{2+}\) influx whereas during HFS protocols that induce LTP the NR1/NR2A mediates the greater total charge transfer and Ca\(^{2+}\) influx (Erreger et al., 2005). This study has demonstrated that the NR2A subunit is critically required in the induction of LTP. Furthermore, it is known that LTP requires protein kinase activation (Malenka et al., 1989) while LTD requires protein phosphatase activation (Mulkey et al., 1993; Mulkey et al., 1994). This study has also shown that the protein phosphatase 2B (calcineurin) inhibitor cyclosporin A is able to prevent the effect of Ro 25-6981. This is consistent with reports of calcineurin being critical in the induction of LTD (Mulkey et al., 1994). This study cannot rule out the involvement of the NR2B subunit in Schaffer collateral-CA1 LTP, especially considering the interaction of NR2B with the critical NR2A subunit-containing NMDA receptors described, the marginal reduction in LTP induced and the many reports implicating the NR2B subunit in LTP.

This report has suggested that NR2B subunit-containing NMDA receptors, which have been reported to be tonically or constitutively active (Sah et al., 1989; Berretta & Jones, 1996; Breukel et al., 1998; Woodhall et al., 2001) and are critically involved in the induction of LTD (Sjostrom et al., 2003),
negatively gate under physiological conditions the NR2A subunit-containing NMDA receptor. It is widely reported and our studies have also found that successful NMDA dependent LTD induction is very difficult to achieve whilst LTP induction is relatively easy (Kemp & Bashir, 2001; Massey et al., 2004). It could be that such tissues, rather than being in a resting state, are in a maximal LTD state and are therefore only able to be potentiated towards an LTP state from which level they can then be easily depotentiated. This could be explained by differential expression of NR2 subunits in NMDA receptors. This study has not demonstrated that there is a way to increase the levels of the NR2B-mediated restraint and this may be because the level of restraint is saturated. A caveat to this link is the exclusivity to the NMDA receptor and the requirement of NMDA agonist to observe this phenomenon. The induction of LTP and LTD critically require the NR2A and NR2B subunits respectively but they are further associated with the trafficking of AMPA receptors to and from the postsynaptic membrane and an associated change in the synaptic weight of neuronal transmission independent of NMDA receptor agonists (Malinow & Malenka, 2002; Watt et al., 2004). This is missing from the interaction this study has shown but if bridged might lead to the induction of LTP proper.
Donald Hebb’s neurophysiological hypothesis states that ‘When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased.’ (Hebb, 1946).

A modification of this hypothesis has led to the Bienenstock-Cooper-Munro (BCM) theory of the sliding threshold of synaptic plasticity (Bienenstock et al., 1982). It hypothesizes that active synapses grow stronger or weaker when postsynaptic activity exceeds or falls below a ‘modification threshold’, \( \theta_m \). Presynaptic activity triggers synaptic depression or potentiation depending on the concurrent level of postsynaptic activity. The history of integrated postsynaptic activity determines the threshold level at which plasticity is induced; the value of \( \theta_m \) varies as a function of the history of integrated postsynaptic activity. As average activity falls or rises, so does the value of \( \theta_m \).

This hypothesis led to the observations in the visual cortex that experience drives NMDA receptor subunit switching from LTD-induction-preferring NR1/NR2B heterodimeric to LTP induction-preferring NR1/NR2B/NR2A heterotrimeric receptors (Kirkwood et al., 1996; Philpott et al., 2001). NR2A and NR2B subunit-containing receptors provide the biological basis for the BCM sliding threshold of synaptic plasticity. This model is further strengthened by the recent reports of NR2A and NR2B antagonist sensitive
induction of LTP and LTD respectively (Liu et al., 2004; Massey et al., 2004; Mallon et al., 2005). The negative coupling of NR2B to NR2A subunit-containing receptors may represent a further mechanism for fine-tuning the strength of NMDA mediated synaptic transmission and for maintaining a threshold for induction of synaptic plasticity that only requires changes in the NR2B subunit. Perhaps by lateral diffusion between synaptic and extrasynaptic sites, in agreement with recent studies where the NR2A subunit does not appear to be very mobile (Groc et al., 2004; Triller & Choquet, 2005). Furthermore, as well as the induction threshold for LTP or LTD induction there is also the magnitude of change in strength elicited (Castellani et al., 2001). It was found in this study that whilst LTD could not be induced, the level of LTP that was reliably induced was of a particularly high magnitude. A level at which it was then possible to depotentiate from (data not shown). It may be that the experience-deprived environment the rats are familiar with, means that the biological basis for the sliding threshold is set such that they are in a maximal state of LTD and that only LTP can be induced. It has been reported that rats from enriched environments are more likely to exhibit LTD (T.V.P. Bliss, personal communication). The investigation of this could help to discover more about the biological basis for the magnitude of synaptic plasticity induced and the effects of environment (Castellani et al., 2001).
Figure 61. Phosphatase hypothesis.

This scheme summarises the intracellular transduction systems that account for the differing pharmacology and physiology of the NR2A and NR2B subunit-containing receptors, the associated phosphoprotein steps and their influence over LTP and LTD. In brackets are the relevant antagonists (Mulkey et al., 1994; Revest & Longstaff, 1998).
Appendix 1

Methods

Experiments were carried out by Professor T.W. Stone to further investigate the actions of Ro 25-6981 using intracellular recording techniques.

Intracellular recordings were made using sharp glass microelectrodes (o.d. 1.5mm) (Clark Electromedical, Harvard Instruments). These were pulled on a Narashige vertical puller in a manually controlled two-stage process to produce pipettes with a shank length of approximately 20mm. The electrodes were filled with 1M potassium acetate, yielding a resistance of 80-100MΩ when measured in the extracellular space of the brain slices via the Neurolog amplifier. Potentials were amplified by a Neurolog 102 DC amplifier or by an Axoclamp-2A operated in bridge balance mode, and filtered between DC and 20KHz. The electrode was advanced in 2.5μm steps using a remotely controlled World Precision Instruments motorised stepper. When a neurone was approached, indicated by a rise in electrical noise, a brief increase of electrode capacitance (‘buzz’) was induced in order to facilitate entry. Cells were allowed to recover from penetration for at least 10 minutes before examining them for electrical properties and stability. Neurones were then used for experimentation if they displayed a
stable resting potential (±10% over 20 minutes) greater than 60mV and a
spike of at least 70mV in response to an intracellular depolarising current
pulse. Current pulses (0.1-1nA) were used to monitor membrane input
resistance throughout the experiments. Records were calibrated using the
amplifier’s own calibration pulse (10mV), and were recorded directly onto
a high frequency thermal chart recorder (Grass instruments Dash IV),
although the filter settings were placed at DC to 10Hz to reduce interfering
transients and action potentials.
Results

NMDA

Four neurones were tested with a combination of NMDA and Ro 25-6981. In each case, NMDA was superfused at a concentration of 2.5uM for 10 minutes, with 20 minutes between each application. At least two control applications were made. At this concentration, the cells showed little or no response, the two applications of NMDA immediately prior to Ro 25-6981 yielding barely discernible depolarisations of 0.19±0.048, 0.26±0.059mV (n=4). Ten minutes after the second of these controls, superfusion of Ro 25-6981 (3uM) was commenced for 20 minutes. The next application of NMDA (parallel with the second 10 minutes of Ro 25-6981) produced a similarly weak effect (0.26±0.066mV). However, the next application, which was begun 20 minutes after ending the superfusion of Ro 25-6981, produced a substantial depolarisation (10.67±1.25mV; p<0.00001, n=4) (figures 62 and 63). This suggests that the negative coupling of NR2B to NR2A subunit-containing NMDA receptors is occurring at postsynaptic sites.
Figure 62. Intracellular recordings of NMDA responses and the effect of Ro 25-6981

Intracellular recordings from a pyramidal neurone of resting potential –74 mV, with input resistance monitored by hyperpolarising pulses of 0.3nA amplitude, 300 ms duration every 20 s. Recordings are from one of six neurons tested with 2.5μM NMDA and 3μM Ro 25-6981. In panels a–c, NMDA was superfused alone at 2.5μM for ten minutes, with twenty minutes between each application. Ten minutes after record c, Ro 25-6981 (3μM) was applied for twenty minutes. The next application of NMDA (d) (parallel with the second ten minutes of Ro 25-6981) still produced no clear response. The next application (e, twenty minutes after ending Ro 25-6981) indicates an increased frequency of excitatory synaptic potentials elicited by NMDA, while in panel (f), NMDA produced a substantial depolarisation with superimposed action potentials (truncated).
Figure 63. Intracellular recordings showing the effect of NMDA alone and after the application of Ro 25-6981.

Intracellular recordings from a pyramidal neurone of resting potential $-68$ mV, with input resistance monitored by hyperpolarising pulses of 0.3$nA$ amplitude, 300 ms duration every 20 s. Recordings are from a second of the six neurons tested with 2.5$\mu$M NMDA and 3$\mu$M Ro 25-6981. NMDA was superfused alone at 2.5$\mu$M for ten minutes, with twenty minutes between three repeated applications (a). Ten minutes after record the third application had ended, Ro 25-6981 (3$\mu$M) was applied for twenty minutes. The next application of NMDA (parallel with the second ten minutes of Ro 25-6981) still produced no clear response. The next application (twenty minutes after ending Ro 25-6981) indicated an increased frequency of excitatory synaptic potentials elicited by NMDA, while in panel b, a further application of NMDA (fifty minutes after ending Ro 25-6981) produced a substantial depolarisation with superimposed action potentials (truncated).
Appendix 2

PEAQX is an NMDA receptor antagonists with a preference for the 1A/2A, rather than 1A/2B receptor composition.


PEAQX is a water-soluble quinoxalinedione with a high affinity for the glutamate-binding site of NMDA receptors ([³H]CGP39653 binding assay: IC₅₀ = 11nM). At a concentration of 10 μM, PEAQX showed no additional activity in a battery of 68 radioligand binding assays for CNS receptors.

In vitro, PEAQX possesses a 126-fold selectivity for the NMDA 1A/2A over 1A/2B receptor composition expressed in Xenopus oocytes (Figure 1, IC₅₀ = 14nM and 1800nM respectively). Experiments on embryonic cortical cells and on neocortical slices confirm these results on native receptors: Cultured embryonic cortical cells express NR2A subunits at a very low level, resulting in a low potency of PEAQX (IC₅₀ = 1 μM). NR2A subunits are expressed to a much larger extent in adult slices, and the effect of PEAQX is therefore stronger in this model (IC₅₀ = 68nM).

Figure 1: Effect of PEAQX on NMDA-induced currents in Xenopus oocytes expressing human NMDAR 1A/2A or 1A/2B. Structure of (1RS, 1'S)-PEAQX.

In vivo, PEAQX is a potent anticonvulsant in both DBA/2 mice (tonic convulsions: ED₅₀ = 1.2 mg/kg, 1h i.p.) and in the mouse maximal electroshock test (ED₅₀ = 5 mg/kg, 1h i.p.), with a long duration of action (≥ 24h). Anticonvulsant activity after intravenous administration indicates a rapid brain penetration. The side effect profile of PEAQX is clearly different from non-selective or NMDA 1A/2B selective NMDA antagonists, as it causes no motor stimulation at anticonvulsant doses, does not disrupt the prepulse inhibition in the rat startle model, and does not substitute for PCP in rats trained in a two lever operant task.

* K-136.6.82, Novartis Pharma AG, 4002 Basel, Switzerland.
Tel. +41 61 696 8404, Fax +41 61 696 8676, yves.auberson@pharma.novartis.com

200
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