Modulation of the AMPA Receptor by Membrane cholesterol and neuroactive steroids

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By

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Abstract

Cholesterol in the plasma membrane of cells has been found to modulate the actions of various proteins in the membrane. This thesis describes the influence of membrane cholesterol on the excitatory AMPA receptor in acutely dissociated rat hippocampal neurones. Non-genomic influences of acute exposure to neuroactive steroids on the function of the AMPA receptor have also been investigated.

Neuronal membrane cholesterol levels were altered by means of the cholestrol carrier methyl-β-cyclodextrin. Using the patch clamp technique, whole cell current responses to local applications of AMPA were recorded. Depletion of cholesterol to 62% control increased the potency of AMPA, whilst enrichment to 205% control decreased the potency of AMPA and the maximum response obtainable. The AMPA responses showed rapid desensitization, which was reduced in cholesterol-depleted neurones and increased in cholesterol-enriched neurones. NBQX antagonised responses to AMPA competitively but failed to affect the relationship between desensitization and AMPA concentration. GYKI 52466 reduced the maximum response to AMPA and shifted the desensitization profile to higher AMPA concentrations.

The neuroactive steroids pregnenolone sulphate, deoxycorticosterone, corticosterone and progesterone reduced the maximum response to AMPA and reduced its potency whereas tetrahydrodeoxycorticosterone, allopregnanolone and epiallopregnanolone simply reduced the potency of AMPA. The 5β isomers of the former two steroids were inactive. Cholesterol depletion enhanced and enrichment diminished the reductions in AMPA potency by all of the active steroids.
Possible mechanisms underlying these novel observations with cholesterol and the neuroactive steroids include membrane fluidity changes and specific modulatory sites for specific steroids on the AMPA receptor protein. These are further discussed.
Chapter 1. General Introduction

1.1 Aims and Objectives .................................................................2
1.2 Plasma membrane of neuronal cells ..........................................3
1.3 Cholesterol and biological membranes .................................4
   1.3.1 Location of cholesterol in the membrane .........................4
   1.3.2 Cholesterol absorption and synthesis .................................7
   1.3.3 Cholesterol effects on the physical properties of the membrane .................................................................8
   1.3.4 Cholesterol influence on the functions of membrane proteins .................................................................9
1.4 Glutamate and excitatory receptors .........................................11
1.5 The AMPA receptor ...............................................................13
   1.5.1 AMPA modulation of cationic channels .............................15
   1.5.2 Divalent permeability .....................................................15
   1.5.3 The number of agonist binding sites ................................16
   1.5.4 Association of glutamate receptors with intracellular proteins .................................................................17
   1.5.5 Internal polyamine block of AMPA receptors .....................18
   1.5.6 AMPA receptor desensitization .......................................19
Chapter 2. Materials and Methods

2.1 Introduction .................................................................42
2.2 Dissociation of Hippocampal Neurones .................................42
2.3 Preparation of methyl-β-cyclodextrin solution and the cholesterol inclusion complex ........................................44
2.4 Preparation of epicholesterol-methyl-β-cyclodextrin inclusion complex ......................................................44
2.5 Manipulation of membrane cholesterol using methyl-β-cyclodextrin and its cholesterol and epicholesterol inclusion complex .......................45
2.6 Cholesterol and protein assay of hippocampal neurones .............46
2.7 Electrophysiological measurements ....................................50
2.8 Construction of a U-tube ....................................................51
2.9 Standardisation of AMPA current responses ..........................54
2.10 Drugs and chemicals ......................................................55
2.11 Data analysis ..................................................................56
2.12 Discussion ......................................................................57
Chapter 3. **Manipulation of Membrane Cholesterol**

3.1 Introduction .................................................................................................60
3.2 The viability of hippocampal neurones ......................................................61
3.3 Alteration of membrane cholesterol content by methyl-β-cyclodextrin and its cholesterol inclusion complex ........................................61
3.4 Effect of time elapsed on membrane cholesterol content of neurones ....................................................................................................65
3.5 Discussion ...................................................................................................66

Chapter 4. **Effect of Membrane Cholesterol on the Sensitivity of AMPA receptors to AMPA**

4.1 Introduction .................................................................................................69
4.2 Effect of membrane cholesterol on AMPA currents .................................70
   4.2.1 Effect of membrane cholesterol on peak AMPA currents ................72
   4.2.2 Effect of membrane cholesterol on plateau AMPA currents ..........75
   4.2.3 Effect of cholesterol on the decay from peak to plateau AMPA currents..................78
   4.2.4 Effect of cholesterol on the rate of current decay from peak ..........79
4.3 Effect of membrane cholesterol on the current-voltage relationship ..........81
4.4 Restoration of membrane cholesterol following depletion .......................83
4.5 Effect of acute application of cholesterol on AMPA currents ...............86
4.6 Discussion ...................................................................................................87
6.11 Discussion

Chapter 7. Effect of Membrane Cholesterol on the Modulation of AMPA Currents by Neuroactive Steroids

7.1 Introduction

7.2 Effect of the manipulation of plasma membrane cholesterol on the modulatory influence of 10 μM pregnenolone sulphate

7.3 Effect of the manipulation of plasma membrane cholesterol on the modulatory influence of 1 μM deoxycorticosterone

7.4 Effect of the manipulation of plasma membrane cholesterol on the modulatory influence of 10 μM THDOC

7.5 Effect of epicholesterol enrichment

7.6 Discussion

Chapter 8. Effect of Membrane Cholesterol on the Modulation of AMPA Currents by Receptor Antagonists

8.1 Introduction

8.2 NBQX antagonism

8.2.1 Effect of NBQX on AMPA currents

8.2.2 Effect of NBQX on the decay from peak to plateau AMPA currents

8.2.3 Effect of the manipulation of plasma membrane cholesterol on NBQX antagonism

8.3 GYKI 52466 antagonism

8.3.1 Effect of GYKI 52466 on AMPA EC50

8.3.2 Effect of GYKI 52466 on the decay from peak to plateau AMPA currents

8.3.3 Effect of the manipulation of plasma membrane cholesterol on GYKI 52466 antagonism
Chapter 9. General Discussion

9.1 The influence of membrane cholesterol on AMPA.................................193
9.2 Comparison with the effect of membrane cholesterol on other receptors...................................................................................................195
9.3 The influence of neurosteroids on AMPA...............................................196
9.4 Cholesterol interaction with modulators of the AMPA receptor............200
9.5 Sites of cholesterol-neuroactive steroid interaction..............................203
9.6 In-Vivo implications of AMPA receptor modulation.............................207
   a) Cholesterol implications....................................................................207
   b) Neurosteroid implications.................................................................210
9.7 Conclusion............................................................................................214

Acknowledgements

References
List of Figures

1.1 Structure of cholesterol (5-cholesten-3β-ol) ......................................................... 4
1.2 Model of synaptic plasma membrane lipid domains ............................................. 6
1.3 Schematic representation of the AMPA receptor subunits ................................. 14
1.4 The proposed domain structure of iGluRs ........................................................... 21
1.5 The chemical structure of DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) ................................................................. 23
1.6 Schematic representation of the Schaffer collateral pathway between the CA3 and CA1 hippocampal neurones which is a glutamatergic excitatory pathway ................................................................. 28
1.7 Potential paths leading to neuronal injury resulting from an episode of ischaemic insult ......................................................................................... 32
1.8 The lettering of the steroid rings and numbering of the carbon atoms in the steroidal structure, showing pregnanolone ............................................. 35
1.9 Outline of the synthetic pathways for neuroactive steroid synthesis in the nervous system ......................................................................................... 36

2.1 True phase photomicrograph of an acutely dissociated hippocampal neurone, prior to cholesterol manipulation procedure ......................................... 43
2.2 Standard curve of cholesterol measured by cholesterol diagnostic kit (Sigma, US) ......................................................................................... 49
2.3 Standard curve of protein measured by BioRad Detergent Compatible Protein Assay kit from BIO-RAD (Germany) ................................................. 49
2.4 Schematic diagram showing the stages of U-tube fabrication process ............... 53
2.5 Schematic diagram showing AMPA receptor response to a 1.0 second application of AMPA in comparison to GABA receptor response to GABA ................................................................................................. 54

3.1 Alteration of membrane cholesterol by methyl-β-cyclodextrin in acutely dissociated hippocampal neurones ................................................................. 63
4.1 The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on AMPA currents in acutely dissociated hippocampal neurones .......................................................... 71

4.2 AMPA dose-response relationships showing the whole cell peak current responses to AMPA recorded from acutely dissociated hippocampal neurones voltage clamped at -40mV ........................................ 73

4.3 AMPA dose-response relationships showing whole cell peak current responses to AMPA recorded from cholesterol-enriched acutely dissociated hippocampal neurones clamped at -40mV .................. 74

4.4 Whole cell plateau current responses to AMPA recorded from cholesterol-depleted acutely dissociated hippocampal neurones clamped at -40mV .................................................. 74

4.5 AMPA dose-response relationships showing whole cell plateau current responses to AMPA recorded from cholesterol-enriched acutely dissociated hippocampal neurones clamped at -40mV .................. 76

4.6 Whole cell plateau current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV .......................................................... 77

4.7 The percentage desensitization of AMPA receptor responses in control, cholesterol-enriched and depleted neurones .............................................. 78

4.8 AMPA (300μM) evoked whole cell currents in acutely dissociated hippocampal neurones showing the degree of decay in response in (A) control neurone vs enriched and (B) control vs depleted neurone. (C) The decay of a control current shown on an expanded time scale, fitted by a single exponential with a time constant ($\tau_D$) .................. 80

4.9 The peak and steady-state current-voltage (I/V) relation of AMPA receptor mediated responses in whole cell recordings of hippocampal neurones .......................................................... 82

4.10 Whole cell peak current responses to AMPA recorded from control, cholesterol-depleted and cholesterol-repleted hippocampal neurones clamped at -40mV .................................................. 84
4.11 The effect of acute application of cholesterol on the AMPA concentration-response curves.................................................................86
4.12 Schematic model demonstrating a possible method for the relief of the conformation-induced bending strain in the lipid bilayer experienced upon binding of the agonist in cholesterol-depleted neurones..............91

5.1 The cholesterol content of neurone samples following incubation at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or epicholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM epicholesterol + 1.5mM MβC (enrichment)..........................................................98
5.2 Whole cell peak current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV.........................100
5.3 Whole cell peak current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV.........................100
5.4 Whole cell plateau current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV..................101
5.5 The percentage desensitization of AMPA receptor responses in depleted and epicholesterol-repleted neurones..........................103

6.1 The structures of the main neuroactive steroids outlined in the chapter........110
6.2 The effect of application of 10μM cortisol on the AMPA concentration-response curve in acutely dissociated hippocampal neurones........111
6.3 AMPA (30 μM) evoked whole cell currents in a control acutely dissociated neurone (A), in the presence of varying concentrations of PS (B-D), and following a washout period of 3 minutes (E)..............114
6.4 AMPA dose-response relationships showing whole cell peak current responses to AMPA alone and in presence of 0.1μM PS, 1μM PS and 10 μM PS, recorded from dissociated hippocampal neurones clamped at -40mV.................................................................116
6.5 The degree of fade to plateau of control AMPA receptors expressed
as the percentage desensitisation in control neurones (dotted line),
and control neurones + 10μM PS.................................................................116

6.6 The peak current-voltage (I/V) relation of AMPA receptor mediated
responses in whole cell recordings of control hippocampal neurones
and in the presence of 10 μM pregnenolone sulphate..............................117

6.7 AMPA (30 μM) evoked whole cell currents in a control acutely
dissociated neurone (A), in the presence of varying concentrations of
deoxyorticosterone (DOC) (B-D), and following a washout period of
3 (E) and 5 minutes (F)..............................................................................119

6.8 AMPA dose-response relationships showing whole cell peak
current responses to AMPA alone and in presence of 0.1μM DOC,
1μM DOC and 10μM DOC recorded from dissociated hippocampal
neurones clamped at -40mV.................................................................121

6.9 The degree of fade to plateau of control AMPA receptors expressed
as the percentage desensitisation in control neurones (dotted line),
and control neurones + 10μM DOC.........................................................121

6.10 AMPA dose-response relationships showing whole cell peak
current responses to AMPA alone and in presence of 20μM
progesterone, recorded from dissociated hippocampal neurones
clamped at -40mV................................................................................124

6.11 AMPA (30 μM) evoked whole cell currents in a control acutely
dissociated neurone (A), in the presence of varying concentrations
of THDOC (B-D), and following a washout period of 3 minutes (E).........127

6.12 AMPA dose-response relationships showing whole cell peak current
responses to AMPA alone and in presence of 0.1μM THDOC, 1μM
THDOC and 10 μM THDOC, recorded from dissociated hippocampal
neurones clamped at -40mV....................................................................129

6.13 The degree of fade to plateau of control AMPA receptors expressed
as the percentage desensitisation in control neurones (dotted line),
and control neurones + 10μM THDOC.....................................................129

6.14 AMPA dose-response relationships showing whole cell peak current
responses to AMPA in presence of 1 and 10μM Allopregnanolone, recorded from dissociated hippocampal neurones clamped at −40mV..........134

6.15 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 10μM epiallopregnanolone, recorded from dissociated hippocampal neurones clamped at −40mV..........135

6.16 AMPA (300μM) evoked whole cell currents in acutely dissociated hippocampal neurones showing the degree of decay of response in the presence of A) pregnenolone sulphate (10μM) and deoxycorticosterone (10μM), B) THDOC (10μM).................................................................136

6.17 AMPA dose-response relationships showing whole cell peak current responses to AMPA for control, in presence of 10μM THDOC, 6μM NBQX and 10μM THDOC + 6μM NBQX recorded from acutely dissociated hippocampal neurones clamped at −40mV..............139

6.18 AMPA dose-response relationships showing whole cell peak current responses to AMPA for control, in presence of 10μM cortisol, 10μM pregnenolone sulphate and 10μM cortisol + 10μM pregnenolone sulphate recorded from acutely dissociated hippocampal neurones clamped at −40mV..................................................139

6.19 Chemical structures of i) 5α- and ii) 5β-THDOC........................................141

7.1 The effect of membrane cholesterol alterations on the concentration-response relationship of peak AMPA currents and their modulation by 10μM pregnenolone sulphate. A) cholesterol depletion. B) cholesterol enrichment.................................................................153

7.2 The effect of membrane cholesterol alterations on the concentration-response relationship of peak AMPA currents and their modulation by 1μM deoxycorticosterone. A) cholesterol depletion. B) cholesterol enrichment.........................................................156

7.3 The effect of membrane cholesterol alterations on the concentration-response relationship of peak AMPA currents and their modulation by 10μM THDOC. A) cholesterol depletion. B) cholesterol enrichment..........159
7.4 The effect of membrane epicholesterol enrichment on the concentration-response relationship for the modulation of peak AMPA currents. 
A) In the presence of 10μM pregnenolone sulphate. B) In the presence of 10μM THDOC

8.1 Peak current responses to 30μM AMPA in the presence of varying concentrations of NBQX, recorded from acutely dissociated hippocampal neurones clamped at -40mV

8.2 AMPA currents in a control (A) acutely dissociated hippocampal neurone, and in the same neurone, in the presence of 6μM NBQX (B)

8.3 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 6μM NBQX, recorded from dissociated hippocampal neurones clamped at -40mV (n=13)

8.4 The degree of fade to plateau of control AMPA receptors expressed as the percentage ratio of steady-state plateau current (Iss) to peak current (Ip) of control neurones and in the presence of NBQX

8.5 The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on the concentration-response relationship for the antagonism of AMPA currents by 6μM NBQX. A) cholesterol enrichment. B) cholesterol depletion

8.6 Peak current responses to AMPA in the presence of varying concentrations of GYKI52466, recorded from acutely dissociated hippocampal neurones clamped at -40mV

8.7 The antagonism of AMPA currents in control acutely dissociated neurones (A) in the presence of 15μM GYKI52466 (B)

8.8 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 15μM GYKI 52466, recorded from dissociated hippocampal neurones clamped at -40mV

8.9 AMPA dose-response relationships showing whole cell plateau current responses to AMPA in presence of 15μM GYKI 52466, recorded from dissociated hippocampal neurones clamped at -40mV
8.10 The degree of fade to plateau of control AMPA receptors expressed as the percentage ratio of steady-state plateau current (Iss) to peak current (Ip) control neurones in the presence of 15μM GYKI52466.............183

8.11 The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on the concentration-response relationship for the antagonism of AMPA currents by GYKI 52466. A) cholesterol enrichment. B) cholesterol depletion.................................................................186

9.1 A proposed model (not to scale) for the possible modulation sites of neuroactive steroids on receptor proteins. ..............................................................206
List of Tables

1.1 Expression of AMPA subtypes in the rat hippocampus ........................................27
1.2 Neurotransmitter receptors affected by neurosteroids ...........................................38

3.1 Alterations of membrane cholesterol by methyl-β-cyclodextrin in acutely dissociated hippocampal neurones ........................................................................................................62
3.2 Time elapsed following the incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol (enrichment) ........................................65

4.1 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 1.5mM MβC (enrichment) ........................................70
4.2 The effect of membrane cholesterol alterations on peak AMPA dose-response relationships of acutely dissociated neurones ........................................73
4.3 The effect of membrane cholesterol alterations on plateau AMPA dose-response relationships of acutely dissociated neurones ........................................76
4.4 The effect of membrane cholesterol on AMPA current decay, shown as the rate constant ($\tau_D$) of a single exponential fit ...........................................81
4.5 The incubation of neurone samples with 5mM methyl-β-cyclodextrin (MβC) (depletion) or depletion followed by enrichment with cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC ............................................84
4.6 The effect of membrane cholesterol depletion followed by enrichment on peak AMPA dose-response relationships of acutely dissociated neurones ........................................................................................................85
4.7 The effect of membrane cholesterol depletion followed by enrichment on plateau AMPA dose-response relationships of acutely dissociated
5.1 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or epicholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM epicholesterol + 15mM MβC (enrichment) ........................................ 97

5.2 The effect of membrane epicholesterol enrichment on peak AMPA dose-response relationships of acutely dissociated neurones ........................................ 99

5.3 The effect of membrane cholesterol depletion and depletion followed by epicholesterol enrichment (10 and 20 minutes) on peak AMPA dose-response relationships of acutely dissociated neurones ........................................ 99

5.4 The effect of membrane epicholesterol enrichment on plateau AMPA dose-response relationships of acutely dissociated neurones .................................. 102

5.5 The effect of membrane cholesterol depletion and depletion followed by epicholesterol enrichment (10 and 20 minutes) on plateau AMPA dose-response relationships of acutely dissociated neurones .................................. 102

6.1 The effect of PS on peak AMPA dose-response relationships of acutely dissociated neurones ................................................................. 115

6.2 The effect of PS on plateau AMPA dose-response relationships of acutely dissociated neurones ................................................................. 115

6.3 The effect of DOC on peak AMPA dose-response relationships of acutely dissociated neurones ................................................................. 120

6.4 The effect of DOC on plateau AMPA dose-response relationships of acutely dissociated neurones ................................................................. 120

6.5 The effect of THDOC on peak AMPA dose-response relationships of acutely dissociated neurones ................................................................. 128

6.6 The effect of THDOC on plateau AMPA dose-response relationships of acutely dissociated neurones ................................................................. 128

6.7 The effect of Allopregnanolone on peak AMPA dose-response relationships of acutely dissociated neurones ................................................................. 132
6.8 The effect of epiallopregnanolone on peak AMPA dose-response relationships of acutely dissociated neurones ....................................................132
6.9 The effect of Allopregnanolone on plateau AMPA dose-response relationships of acutely dissociated neurones .....................................................133
6.10 The effect of epiallopregnanolone on plateau AMPA dose-response relationships of acutely dissociated neurones .....................................................133
6.11 The effect of neurosteroids on AMPA current decay, shown as the rate constant of a single exponential fit \( (\tau_D) \) .......................................................135
6.12 The effect of combined presence of 10 \( \mu \)M THDOC and 6 \( \mu \)M NBQX on AMPA EC\(_{50}\) values, compared to individual exposures ..................137

7.1 The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 10\( \mu \)M pregnenolone sulphate in acutely dissociated neurones ...........................................................................152
7.2 The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 10\( \mu \)M pregnenolone sulphate in acutely dissociated neurones ...........................................................................152
7.3 The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 1\( \mu \)M deoxycorticosterone in acutely dissociated neurones ...........................................................................155
7.4 The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 1\( \mu \)M deoxycorticosterone in acutely dissociated neurones ...........................................................................155
7.5 The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 10\( \mu \)M THDOC in acutely dissociated neurones ...........................................................................158
7.6 The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 10\( \mu \)M THDOC in acutely dissociated neurones ...........................................................................158
7.7 Comparison of the effect of 10 \( \mu \)M pregnenolone sulphate on AMPA peak responses in control, cholesterol-enriched and epicholesterol-
7.8 Comparison of the effect of 10 μM THDOC on AMPA peak responses in control, cholesterol-enriched and epicholesterol-enriched neurones........................................................................................................161

8.1 The effect of NBQX on peak AMPA dose-response relationships of acutely dissociated neurones........................................................................................................172

8.2 The effect of NBQX on plateau AMPA dose-response relationships of acutely dissociated neurones..................................................................................172

8.3 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC (enrichment).......................................................174

8.4 The effect of membrane cholesterol enrichment on antagonism of peak AMPA currents by NBQX in acutely dissociated neurones.....................................176

8.5 The effect of membrane cholesterol depletion on antagonism of peak AMPA currents by NBQX in acutely dissociated neurones.....................................176

8.6 The effect of membrane cholesterol enrichment on antagonism of plateau AMPA currents by NBQX in acutely dissociated neurones..................................178

8.7 The effect of membrane cholesterol depletion on antagonism of plateau AMPA currents by NBQX in acutely dissociated neurones..................................178

8.8 The effect of GYKI52466 on peak AMPA dose-response relationships of acutely dissociated neurones.................................................................181

8.9 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC (enrichment).......................................................185

8.10 The effect of membrane cholesterol enrichment on antagonism of peak AMPA currents by GYKI52466 in acutely dissociated neurones..................................185

8.11 The effect of membrane cholesterol depletion on antagonism of peak AMPA currents by GYKI52466 in acutely dissociated neurones..................................185
8.12 The effect of membrane cholesterol enrichment on antagonism of plateau AMPA currents by GYKI 52466 in acutely dissociated neurones...188

8.13 The effect of membrane cholesterol depletion on antagonism of plateau AMPA currents by GYKI 52466 in acutely dissociated neurones.............188
List of Publications


Chapter 1

General Introduction
Chapter 1

1.1 Aims and objectives

Although much interest has been focused in the public domain on the association of cholesterol to conditions of atherosclerosis and its sequelae, cholesterol is, of course, an essential component of all mammalian cells. In neurobiology, three quite different roles of cholesterol can be discerned: as a structural component of plasma membranes of neurones and glia; as a precursor for bile acids and lipoproteins as well as a cascade of steroidal hormones and mediators affecting both genomic and non-genomic processes in neurones; and as a direct modulator of the functions of certain plasma membrane proteins.

The work in this thesis concentrates on the role that membrane cholesterol and steroidal metabolites exert on the AMPA subtype of the glutamatergic system. Detailed functional studies on such interactions involving the AMPA receptors have not, to my knowledge, been previously reported.

The aim of this project has therefore been to investigate the AMPA receptor response under various conditions. These studies were hoped to yield:

i) An understanding of the relationship between neuronal bilayer composition and structural properties, together with their associated influences on AMPA receptor behaviour which may then be developed to yield information about regulatory mechanisms at work in biomembranes.

ii) A more extensive research on the non-genomic influence of neuroactive steroids on the quantitative pharmacological properties of a native population of AMPA receptors.
1.2 Plasma membrane of neuronal cells

The plasma membrane of the cell is a highly differentiated structure which acts to separate the cell from the environment and affords a reactive interface between the external environment and the cytoplasm. The associated specific molecular pumps and gates allow a highly selective permeability barrier which regulates the molecular and ionic composition of the intracellular medium. All membranes contain a substantial proportion of phospholipids, such as phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, and phosphatidylinositol. Cholesterol is especially abundant in the mammalian plasma membrane. Rat synaptic plasma membranes consist of phospholipids, representing about 74% of the total membrane lipids, and cholesterol about 19% of the total membrane lipids (Cotman et al., 1969). The ratio by weight of protein to lipid in neuronal plasma membranes is close to 1.0. This ratio is 0.9 in rat synaptic plasma membrane (Cotman et al., 1969).

In 1972, S. Jonathan Singer and Garth Nicolson proposed a fluid mosaic model for the overall organization of biological membranes. The essence of their model is that for most phospholipids and glycolipids in aqueous media, the favoured structure is a bimolecular sheet rather than a micelle because their two fatty acyl chains are too bulky to fit into the interior of a micelle. The crucial property of this molecular assembly is the bilayer fluidity that assures sufficient lateral mobility of the membrane components to support their biological function.

Towards the cell interior, the lipid bilayer is attached to a cytoskeleton of extended polymeric filaments that are important for maintaining the shape of the cell. On the
exterior side, the membrane is covered by a glycocalyx that is made up by the carbohydrate moieties of membrane proteins and lipids in the form of glycolipids, such as gangliosides, or glycoproteins. The interactions between the molecules of the bilayer, such as lipid-lipid, lipid-protein and lipid membrane-cytoskeletal, lead to a lateral organisation of the membrane often characterized by a substantial degree of static and dynamic membrane heterogeneity (Mouritsen and Biltonen, 1993).

1.3 Cholesterol and biological membranes

1.3.1 Location of cholesterol in the membrane

Cholesterol is one of a family of neutral lipids with a common chemical structure: the steroid ring system. Sterols are found in most plant and animal cells. However, each of these phyla requires its own specific sterol. In the animal kingdom, including humans, cholesterol is the most common essential sterol (Yeagle, 1991).

The nature of the cholesterol molecule is dominantly hydrophobic, with the exception of the hydroxyl groups (Figure 1.1). Therefore, as is typical for amphipathic compounds, cholesterol must be found in hydrophobic environments with a polar interface, such as is offered by lipid bilayers: both simple bilayers and lipid bilayers that are part of the cell membranes.

![Figure 1.1 Structure of cholesterol (5-cholest-3β-ol)](image-url)
Establishing that as much as 90% of the total cellular cholesterol is found in the plasma membrane of the cell (Lange and Ramos, 1983), therefore means that when searching for the role of cholesterol in mammalian cells, the focus of such investigation would lie fundamentally on the influence of cholesterol on plasma membrane properties and functions.

The largest pool and concentration of cholesterol in the body is found in the brain. Being a constituent of myelin and cell membranes, cholesterol is important for the function of this organ and inborn defects in cholesterol synthesis or metabolism such as Niemann-Pick C disease are associated with serious neurological and mental dysfunction (Tint et al., 1994; Wood et al., 1999).

Different types of brain membrane lipid domains, dissimilar in location, structure and function have been described and those domains are shown in Figure 1.2. The exofacial or outer leaflet and the cytofacial or inner leaflet are two major domains of plasma membrane. The lipids in the plasma membrane show a clear asymmetric arrangement, with the majority of the cholesterol found in the cytofacial rather than exofacial leaflet (Devaux, 1991; Zachowski, 1993; Liscum and Underwood, 1995), although many questions still remain as to the mechanisms responsible for generating and maintaining this organization (Wood et al., 1999; Pomorski et al., 2001). Lateral lipid domains comprise a group that includes lipid rafts, caveolae, annular lipid domains, and cholesterol pools.
Figure 1.2 Model of synaptic plasma membrane lipid domains (adapted from Wood et al., 2002): C, cholesterol; GPI, glycosylphosphatidylinositol-anchored protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

Cholesterol can move (flip/flop) between outer and inner leaflets of the membranes with a migration time ranging from a few minutes ($t_{1/2}$ between 1-6 minutes) in biological membranes (Schroeder et al., 1991) to several hours ($t_{1/2}$ between 9-38 hours) in liposomal membranes (Rodrigueza et al., 1995).

Sterols are also components of membrane domains (rafts) that may serve as platforms for signaling molecules as well as devices required for the sorting of vesicular cargo (Jacobson and Dietrich, 1999; Brown, 2000; Simons et al., 2000). Cells compensate for sterol excess by efflux pathways (Rothblat et. al, 1999; Liscovitch and Lavie, 2000) in which sterols are captured by acceptors in the extracellular milieu, or via enzymatic esterification reactions (Yang et al., 1996) that result in the more hydrophobic sterol 3-hydroxy fatty acyl esters being sequestered away from bulk membranes in intracellular
lipid particles. De-esterification processes come into play to release sterol to the various membrane systems as needed (Menon, 2002).

1.3.2 Cholesterol absorption and synthesis

Sterols such as cholesterol are widely found to be a requirement for cell growth and differentiation. They are synthesized according to long, complicated, and energetically expensive pathways, independent of those for common lipids. To synthesize cholesterol from acetyl-CoA requires about 30 enzymatically catalyzed steps, an extensive use of reducing equivalents (NADH and NADPH), a number of enzymes and co-factors (all of which must be synthesized by the cell), and molecular oxygen. The expense of such a considerable amount of cellular energy would lead to the deduction that sterols are crucial to cell survival, and that only a particular sterol structure is maximally useful to a particular cell type (Yeagle, 1993). Yet the accumulation of the mammalian sterol, cholesterol, in the form of cholesterol ester and free cholesterol in atherosclerotic plaques is deadly to the human organism. Thus, there is an apparent dichotomy between the essential and lethal natures of this natural compound.

In humans, dietary cholesterol is effectively absorbed, while dietary sitosterol, campesterol, and stigmasterol are not efficiently absorbed (less than 10% of the absorption efficiency of cholesterol) (Yeagle, 1993). There is obviously a selectivity for sterols based on their chemical structure, even though they are all largely hydrophobic and will partition into membranes.

Cholesterol cannot substitute for ergosterol in an ergosterol-requiring cell (for example, yeast), and ergosterol cannot substitute for cholesterol in a mammalian cell. It is
reasonable to suggest, therefore, that while the important roles, which sterols play in cell biology, may be analogous from one cell type to another, finely tuned recognition systems are involved so that subtle changes in the chemical structure of the sterol, render the compound incompetent for its essential role in a foreign cell type.

Mammalian cells obtain cholesterol by internalization of low density lipoproteins (LDL) following receptor-mediated endocytosis and subsequent hydrolysis in lysosomes (Brown and Goldstein, 1986) or by de novo biosynthesis in the endoplasmic reticulum (Reinhart et al., 1987; Neufeld et al., 1996).

Very little cholesterol is taken up into the brain from circulating lipoproteins due to the efficient blood-brain barrier (Dietschy, et. al, 1993). The local synthesis of cholesterol in brain cells is also very low, and it has been reported that only about 0.1% of newly synthesized cholesterol in adult monkey is present in the brain (Spady & Dietschy, 1983). If this is also valid in adult humans, only 1-2 mg of cholesterol would be synthesized each day. From in vitro experiments on slices of rat brain, it was calculated that the half-life of cholesterol is about 6 months (Andersson et.al, 1990). However, even the very low uptake and synthesis of cholesterol in the brain must be balanced by some mechanism for removal of cholesterol. Although very little high-density lipoprotein-dependent cholesterol transport occurs, it must be noted that some of the cholesterol is also utilized for conversion to other metabolites.

1.3.3 Cholesterol effects on the physical properties of the membranes

The presence of cholesterol in the mammalian membranes reduces the conformational flexibility of the hydrocarbon chains of the phospholipids, causing them to adopt an
average conformation in which most carbon-carbon single bonds are in the trans (more ordered) configuration (Yeagle, 1991). The increase in the ordering of the lipid hydrocarbon chains leads to more effective packing of the lipids in the bilayer and consequent reduction in the bilayer packing defects.

The presence of sterols in phospholipid bilayers, therefore, profoundly alters bilayer properties resulting in the retention of a fluid-like microviscosity over a wide temperature range, elimination of the gel-liquid phase transition, and reduction in membrane permeability to small molecules. These effects are particularly evident in the plasma membrane of eukaryotic cells, since these membranes contain the highest sterol concentration of all cellular membranes (Menon, 2002).

The structural requirements for a sterol to produce this special ordering effect include a planar steroid ring system, a hydroxyl group at position 3β, and a hydrophobic cholesterol-like tail at position 17 (Demel et al., 1972).

1.3.4 Cholesterol influence on the functions of membrane proteins

Cholesterol is found in varying proportions in different membranes. Compositional heterogeneity of the lipid fraction of biological membranes, in this case neurones, therefore offers a means for regulating the physical properties of the bilayer, which in turn, can have an influence in mediating the functional responses of membrane proteins. It is hence of interest to characterize, in a controlled manner, the influence of variation in bilayer cholesterol content on the membrane receptor protein channels.

Variations in the composition of cell membranes can strongly affect the behaviour of membrane proteins. The underlying interactions between bilayer components (lipids or
other membrane soluble molecules) and proteins can be distinguished on the basis of "specificity": the former can influence the latter either through direct binding to localized protein sites, or indirectly, by altering the structural, thermodynamic, or dynamic properties of the bilayer, which in turn modulates protein behaviour (Cantor, 1999).

Several membrane receptor proteins have been reported to depend upon cholesterol for their functional activities, for example, the nicotinic acetylcholine receptor (Jones and McNamee, 1988; Fernandez-Ballester, et. al, 1994), the GABA<sub>A</sub> receptor (Sooksawate & Simmonds, 1998), neuronal oxytocin receptors (Gimpl et al., 1997), and rhodopsin (Mitchell et al., 1990; Albert et al., 1996). Other plasma membrane proteins have also been reported to be modulated by cholesterol, such as, the Na<sup>+</sup>-K<sup>+</sup> ATPase, (Yeagle et al., 1988; Vermuri and Philipson, 1989), the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger (Vermuri and Philipson, 1989), adenylyl cyclase (Whetton et al., 1983), the GABA transporter and the L-glutamic acid transporter from rat brain (Shouffani and Kanner, 1990). Cholesterol likely modulates the function of these membrane proteins by more than one mechanism, e.g. alteration of the bulk biophysical properties such as membrane fluidity and/or by a specific cholesterol-protein interaction (Yeagle, 1993; Sooksawate and Simmonds, 1998). Cholesterol alters membrane physical properties by increasing the ordering of the lipid hydrocarbon chains of phospholipids and, thereby, reducing the free volume available to membrane proteins for the conformational changes that may be necessary for binding to specific sites on the membrane proteins or have an influence at the lipid-protein interface to modulate protein function. Those latter mechanisms are likely to be more protein-specific.
1.4 Glutamate and excitatory receptors

Glutamate receptors are the primary excitatory neurotransmitter receptors in the mammalian CNS. They are classified according to the signal transduction mechanism, with metabotropic glutamate receptors operating through second messenger systems and ionotropic glutamate receptors (iGlurRs) as ligand-gated cation channels which convey most of the fast excitatory transmission.

The role of these receptors in the CNS development and in forms of synaptic plasticity underlying learning and memory has also been recognised (Maren & Baudry, 1995; Asztely & Gustafsson, 1996; Turrigiano, 2000). The excitatory amino acid receptors have been implicated in a variety of neurological disorders such as epilepsy, ischaemic brain damage, and more speculatively, neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases, Huntington’s chorea, and amyotrophic lateral sclerosis (Dingledine et al., 1999).

The iGluRs have been classified into three broad subtypes based on pharmacological and electrophysiological criteria: DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (Monaghan et al., 1989; Hollman and Heinemann, 1994; Fletcher and Lodge, 1996).

In terms of functional assignments, it is recognized that AMPA receptors mediate most of the rapid excitatory neurotransmission. NMDA receptor channels display a prominent voltage-dependent Ca$^{2+}$ permeability that is thought to be a prerequisite for use-dependent synaptic plasticity. Considerable evidence for a mutual interaction between kainate and AMPA at their receptor sites has accumulated. It is now clear that where the
conductance response evoked by AMPA has been analysed, it is competitive with kainate, not additive.

Hydrophobicity plots predict four membrane spanning segments in the subunits of the iGluRs, but these subunits are about twice as large as those of the nicotinic, GABA$_A$, glycine, and 5HT$_3$ receptors, and clearly form a separate family. They have a large extracellular amino terminal domain that probably contributes to the ligand-binding site, and the region of M2 is not believed to span the membrane completely, but rather dip into the membrane and return to form a potassium-channel-like pore domain (Figure 1.3). According to this model, the large loop between M3 and M4 is extracellular, whereas it is thought to be intracellular in the other ligand-gated ion channels.

Both kainate and AMPA receptors possess low-affinity binding sites for the endogenous transmitter glutamate, and thus are thought to deactivate quickly because of the brief mean boundtime of the agonist. AMPA receptors, however, recover from desensitization with time constants that are approximately 10-fold faster than kainate receptors (Dingledine et al., 1999).

The focus of this project has been on the AMPA subtype of the glutamate receptors.
1.5 The AMPA Receptor:

AMPA receptors are widespread throughout the CNS and believed to serve as synaptic receptors for fast glutamate-mediated excitatory transmission (Mayer and Westbrook, 1987; Nicoll et al., 1990; Bettler and Mulle, 1995).

Molecular cloning techniques have shown that the AMPA receptor family is composed of four different subunits designated GluR1-4 or GluRA-D (Hollmann et al., 1989; Keinänen et al., 1990). AMPA receptors are most likely to be tetramers that are generated by the assembly of one or more of these subunits, yielding homomeric or heteromeric receptors (Hollmann et al., 1989; Hollmann and Heinemann, 1994; Rosenmund et al., 1998). Additional complexity among AMPA receptors is conferred by alternative splicing of RNA for each subunit, giving rise to flip and flop variants, and post-transcriptional, pre-mRNA editing of nucleotides that code for specific amino acids in the receptor protein. For example, the desensitization of AMPA receptors is influenced by alternative splicing involving a 38 amino acid sequence within the flip/flop region (Sommer et al., 1990; Seeburg et al., 1998), and by the presence of an arginine versus a glycine residue at the site near the flip/flop region (Figure 1.3). Differences in subunit assembly, splice variant expression and editing status give rise to receptors with diverse biophysical and pharmacological properties (Fletcher and Lodge 1996; Bleackman and Lodge, 1998).
Figure 1.3  Schematic representation of the AMPA receptor subunits; adapted and modified from: Dani and Mayer (1995). 1: Flip/flop alternative splice site - a 38 amino acid residue region varies in all AMPA receptor subunits as a result of alternative splicing. 2: Long/short alternative splice site GluR2 and GluR4 undergo alternative splicing to give isoforms with differing C-terminus domains. 3: RNA editing site - edited GluR2 has an arginine residue at this position. Unedited AMPA receptor subunits have a glutamine residue.
1.5.1 AMPA Modulation of Cationic Channels

Relatively little is known about gating mechanisms, other than that they involve a conformational change in allosteric protein channel structure giving rise to different functional properties such as open, refractory or closed states. Neurotransmitter-gated ion channels are regulated by the non-covalent bonding of the ligand which binds to the extracellular side of the channel. The energy from ligand binding drives channel gating towards an open state.

AMPA and kainate directly gate a low-conductance cation channel (less than 20pS) that is permeable to Na\(^+\) and K\(^+\), but less so to Ca\(^{2+}\) ions.

1.5.2 Divalent Permeability

Homomeric and heteromeric channels assembled from GluR1, GluR3, and/or GluR4 subunits show high permeability to Ca\(^{2+}\) (and hence demonstrate rectification), whereas the Ca\(^{2+}\) permeability of channels containing the GluR2 subunit is low (Hollmann et al., 1991; Verdoorn et al., 1991).

The current-voltage (I-V) relations measured for homomeric GluR-4 channels in high Na\(^+\) extracellular solution in the presence of different Ca\(^{2+}\) or Mg\(^{2+}\) concentrations indicate that relatively small changes in extracellular divalent concentration may suffice to change the rectification of the whole-cell current. Heteromeric channels may co-exist in the same cell with homomeric channels with high divalent permeability, suggesting that the glutamate-activated divalent permeability of single cells may represent the superposition of currents mediated by a combination of both types of AMPA receptor channels (Burnashev et al., 1992). AMPA receptor subunits co-assembled with the
edited GluR2 (arginine) subunit exhibit a linear or outwardly rectifying I-V relationship, while recombinant receptors lacking the GluR2 subunit exhibit strong inward rectification (Boulter et al., 1990; Keinänen et al., 1990; Herb et al., 1992). Several data indicate that a reduction in edited GluR2 subunit expression with subsequent formation of Ca\(^{2+}\)-permeable AMPA receptor is likely to be a major factor contributing to the delayed neurodegeneration that follows global ischaemia and kainate-induced status epilepticus (Pellegrini-Giampietro et al., 1992; Fujisawa et al., 1993; Jonas et al., 1994). As such, drugs that selectively block GluR2-lacking AMPA receptors may be potential therapeutic agents.

1.5.3 The number of agonist binding sites

One aspect of receptor activation that has structural implications is the number of agonist molecules which must bind in order to open the receptor channel. This value can be obtained for non-NMDA receptors by analysis of dose-response data in terms of the Hill equation, if interference by desensitization effects is excluded (Patneau and Mayer, 1990). Unlike the nicotinic acetylcholine receptor (nAChR) and the GABA\(_A\) receptor, which require occupancy by two agonist molecules to open the associated channel (Trussell et al., 1989; Clements et al., 1998), there is evidence that binding of glutamate to a single GluR subunit is sufficient for channel opening, although opening to larger conductance states require the binding of agonist to more than one subunit (Rosenmund et al., 1998).

Individual AMPA subunits are also able to gate independently of the state of other subunits in the tetramer, indicating that activation may differ fundamentally from
desensitization, the latter being a process that appears to require a concerted conformational change involving all four subunits (or both dimers) (Robert et al., 2001). Additionally, the number of agonist binding sites available have been reported to be regulated by large inhibitory proteins. This is consistent with the data using Xenopus brain membranes, where there is an increase in the AMPA affinity upon purification. The existence of such regulatory proteins could explain why AMPA $K_d$ values become much lower by various membrane treatments and during purification (Barnard and Henley, 1990).

### 1.5.4 Association of Glutamate Receptors with Intracellular Proteins

A variety of intracellular proteins that bind to glutamate receptors have recently been described. They appear to be structurally and functionally quite important not only for agonist binding, receptor targeting or clustering, but also in receptor signaling pathways. AMPA receptor targeting and clustering are regulated during development (Rao et al., 1998), depend on synaptic activity, and may also play a role in long term potentiation (LTP). For example, tetanic stimulation of hippocampal slice cultures induce long-lasting AMPA receptor clustering observed by introduction of recombinant GluR1 tagged with green fluorescent protein (Shi et al., 1998). The yeast two-hybrid system has been instrumental in the initial identification of several glutamate receptor-associated proteins, including proteins containing PDZ domains [eg. Proteins of the PDS-95 family, GRIP, AMPA receptor-binding protein (ABP)].

Some PDZ domains, which are 90-amino acid repeats that are known to be involved in protein-protein interactions, are associated with the C termini of certain AMPA and
NMDA receptor subunits. Coimmunoprecipitation has confirmed the association of glutamate receptors with PDZ domain-containing-proteins and signalling molecules (Src, calmodulin, G proteins). Some of these proteins compete for binding to the receptor (often dependent on the calcium concentration) and in some cases binding can be regulated by phosphorylation (Dingledine et al., 1999).

1.5.5 Internal Polyamine Block of AMPA Receptors

Similar to Mg\(^{2+}\) block of NMDA channels, calcium permeable kainate and AMPA receptors are tonically blocked at resting membrane potentials by cytoplasmic polyamine ions (Bowie et al., 1998; Rozov et al., 1998). The initial observations demonstrating that freely diffusible polyamines produce strong voltage-dependent block has provided a molecular understanding of the complex rectification of native and recombinant calcium permeable kainate and AMPA receptors. Bound and unbound polyamines are found in millimolar amounts in virtually all eukaryotic and prokaryotic cells (Watanabe et al., 1991). Most naturally occurring polyamines, such as spermine, spermidine, and putrescine, form complexes with nucleic acids, proteins, and phospholipids, which have implicated them in cell growth and differentiation (Pegg, 1986).

The presence of freely diffusible polyamines in the cytoplasm (5-100\(\mu\)M for spermine and spermidine (Watanabe et al., 1991), has been shown to exert a profound effect on the gating properties of a number of ion channel families, including the non-NMDA receptors (Nicols and Lopatin, 1997; Williams, 1997a,b; Bowie et al., 1999).
1.5.6 AMPA Receptor Desensitization

Ligand-gated and voltage-gated channels enter refractory states through different processes. Ligand-gated channels such as AMPA can enter the refractory state when they are exposed to a high concentration of the ligand. This process is called desensitization and it represents a state in which the neurotransmitter remains bound but the associated ion channel is closed or inactive (Jones and Westbrook, 1996; Rosenmund and Mansour, 2002). Even though desensitization is ubiquitous in receptor-signal-transduction cascades, the molecular mechanisms underlying desensitization of ligand-gated ion channels are poorly understood.

Although in some channels this refractory period occurs due to phosphorylation of the channel molecule by a protein kinase, desensitisation appears to be an intrinsic property of the AMPA-gated channels. This phenomenon is thought to shape the synaptic response and serves a functional importance by prevention of neuronal damage during excitation (Jones and Westbrook, 1996; Stern-Bach et al., 1998).

Although desensitization is of great importance with regards to in-vitro studies of AMPA receptors, at hippocampal synapses in vivo, deactivation, which occurs in the absence of agonist, may be considered the primary determinant of the decay of the synaptic response (Colquhoun et al., 1992; Jonas and Sackmann, 1992). This is because in vivo glutamate released from a nerve terminal is likely to reside in the synaptic cleft for a period of milliseconds, fading well before pronounced AMPA receptor desensitization occurs (Clements et al., 1992). It has however been reported that receptors with very rapid desensitization rates may still enter desensitized states, and
continue to desensitize after removal of agonist, as recovery occurs after a delay (Colquhoun et al., 1992; Hestrin, 1992, 1993). There is also a role for desensitization in the modification of synaptic strength during repetitive firing (Raman and Trussell, 1995).

In whole-cell voltage-clamp recordings, the most common response in cortical or hippocampal neurones is a rapidly desensitizing inward current response to sustained application of AMPA and a non-desensitizing response to sustained application of kainate (Kiskin et al., 1986; Trussell et al., 1988; Thio et al., 1991). Hence upon fast application of AMPA, the agonist elicits currents at the AMPA receptor channels which exhibit a fast rise-time and then decay to a plateau in the continued presence of the agonist. This plateau is more pronounced with flip- than with flop-containing receptors (Sommer and Seeburg, 1992; Fletcher and Lodge 1996).

Variation in subunit composition has been used to explain the significant differences in the rapidity and completeness of AMPA receptor desensitizations observed (Trussell et al., 1988; Tang et al., 1989; Jonas and Sakmann, 1992; Raman and Trussell, 1992).

A plethora of research has, in recent years, been aimed at a better understanding and hence control of AMPA receptor desensitization and its modulation by drugs. This may provide a rational basis for the development of new drugs to downregulate receptor activity during episodes of hyperexcitability such as epilepsy (Rogawski, 1993), or to upregulate glutamate receptor activity, enhancing learning, and alleviating memory loss associated with glutamatergic neurones after stroke or brain injury (Yamada, 1998).
Recent literature suggests that the agonist-binding domain of the AMPA receptor is formed by two globular lobes (domains 1 and 2) consisting of highly ordered α-helices and β-sheets that are connected by two polypeptide strands (crossovers 1 and 2) (Armstrong et al., 1998; Paas, 1998; Armstrong and Gouaux, 2000). This bi-lobed agonist binding domain is illustrated in figure 1.4. Domain 1 is made up of residues mostly from the ‘S1’ region upstream of membrane segment 1 (M1), whereas domain 2 is made up mostly from the ‘S2’ region between M3 and M4 (Armstrong et al., 1999; Partin, 2001). It is believed that the amino acid residues within the S1/S2 structure interact with the flip/flop segment to control desensitization and its modulation by drugs (Partin, 2001).

**Figure 1.4** The proposed domain structure of iGluRs, adapted and modified from Sun et al., 2002; showing the S1 and S2 segments in turquoise and pink, respectively.

Using structural analysis of AMPA receptor binding domains (Armstrong et al., 1998; Armstrong and Gouaux, 2000), it has been suggested that amino acid residues critical in
determining AMPA receptor desensitization and located at subunit-subunit interfaces are not conserved among kainate receptors. Also, single channel analysis of AMPA receptors has been used to suggest that individual subunits may operate in an independent manner, during activation (Rosenmund et al., 1998; Smith et al., 2000). Using crystallographic techniques on wild-type and mutant receptors, a structural explanation using the concept of AMPA receptor dimerisation has also been offered, suggesting that desensitization may occur through the rearrangement of the receptor dimer interface, which disengages the agonist-induced conformation change in the ligand-binding core from the ion channel gate (Sun et al., 2002).

Studies indicate that more than one AMPA receptor subtype can coexist within the same neuron. AMPA receptor diversity is even more extreme, as it appears that subunit stoichiometry is not fixed for AMPA receptors (Washburn et al., 1997). Also, strong evidence supports the existence of an intrinsic modulatory site on individual AMPA receptor subunits (Partin et al., 1994; 1995) through which the compound cyclothiazide acts to reduce desensitization of AMPA receptors (Yamada and Tang, 1993; Patneau et al., 1993). The desensitisation can also be suppressed by lectins, either partially by concavalin A, or completely by pre-treatment with wheatgerm agglutinin (Barnard and Henley, 1990).
1.5.7 Chemical modulators of AMPA receptors

Both NMDA and non-NMDA receptors are activated by the endogenous transmitter, L-glutamate, whereas the putative transmitter candidate, L-aspartate, appears to activate NMDA receptors exclusively (Patneau and Mayer, 1990).

The discovery that the compound quisqualate can activate phospholipase-coupled metabotropic receptors (Sladeczek et al., 1985); and that AMPA (Figure 1.5) was a more selective quisqualate-like agonist actually led to the renaming of the non-NMDA receptors as AMPA and kainate receptors (Collingridge & Lester, 1989).

![Figure 1.5 The chemical structure of DL-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA).](image)

A number of structural analogous of AMPA have been described (Hansen and Krogsgaard-Larson, 1990). These analogues include (RS)-2-amino-3-(3-carboxy-5-methyl-4-isoxazole) propionic acid (ACPA) and (RS)-2-amino-3-(3-hydroxy-5-trifluoromethyl-4-isoxazolyl) propionic acid (Tri-F-AMPA). Electrophysiological studies of native AMPA receptors expressed in cultured hippocampal neurones, have shown the order of potencies as ACPA>AMPA>Tri-F-AMPA (Bank and Lambert, 1999).
A number of allosteric modulators that affect the process of desensitization and deactivation have been characterized in detail. These include the benzothiazides such as cyclothiazide, diazoxide and IDRA 21 (Patneu et al., 1993; Yamada and Tang, 1993), the pyrrolidinone analogs aniracetam, piracetam (Isaacson and Nicoll, 1991; Gouliaev and Senning, 1994; Partin et al., 1996). Aniracetam is not a very potent AMPA receptor modulator, so consequently similar, yet distinct compounds, benzoylpiperidines and benzoylpyrrolidines (frequently referred to as ampakines), which retain the potentiating actions on AMPA receptors were developed (Staubli et al., 1994). The benzoylpiperidines (e.g. 1-(1,3-benzodioxol-5-ylcarbonyl)-piperidine or 1-BCP, and 1-(quinoxalin-6-ylcarbonyl)piperidine, also called BDP-12 or CX516) are not metabolized to an inactive form like aniracetam and are able to enter the CNS in an active form (Staubli et al., 1994). These AMPA modulators have been subjected to extensive in vivo evaluation in rodents and humans and improve performance on a variety of memory tasks (Lynch et al., 1997; Ingvar et al., 1997).

The mechanistic differences between thiazide-related and benzoylpiperidine-related AMPA receptor modulators suggested that these two classes of agents probably act at different allosteric modulatory sites on AMPA receptors (Figure 1.6). To add to the complexity of allosteric modulation of AMPA receptors, there was an important observation that cyclothiazide and the AMPA selective, noncompetitive 2,3-benzodiazepine antagonist GYKI 52466 had opposing actions on AMPA receptor-mediated ionic currents. This raised the possibility of a common allosteric modulatory site on the AMPA receptor that was positively modulated by cyclothiazide and
negatively modulated by 2,3-benzodiazepines (Zorumski et al., 1993). Although subsequent binding, mutational, and physiological experiments indicated that the sites of action of these two drugs are not identical, there is an allosteric interaction between them that accounts for their opposing actions (Johansen et al., 1995; Yamada and Turetsky, 1996; Partin and Mayer, 1996). These observations point to the variety of modulatory targets with potentially complex interactions possessed by AMPA receptors and prompt the as yet unanswered question of whether or not there are endogenous modulators that act upon these sites.

From the diverse barbiturate family, phenobarbitone has been found to block GluR2 AMPA receptors, with a weaker blocking action at GluR1, 3 and 4 (Fletcher and Lodge, 1996).

1.6 AMPA Receptors and the Hippocampus

In this study hippocampal neurones were selected because of their low spontaneous spike activity, prominent response to iontophoretic application of AMPA and high density of AMPA receptors in this specific area of the CNS. Hippocampal neurones also express comparatively lower levels of kainate receptors (Boulter et al., 1990; Herb et al., 1992; Kainänen et al., 1990).

The major excitatory input to the hippocampus originates in the entorhinal area (perforant path) and projects to granule cells of the dentate gyrus (DG). Removal of the entorhinal cortex protects hippocampal CA1 neurones from ischaemic damage (Jorgensen et al., 1987) and reduces stress-induced CA3 atrophy (Sunanda et al., 1997).
In addition, glutamate is the main neurotransmitter in the trisynaptic loop of the hippocampal formation (DG-CA3-CA1-subiculum) (Vizi and Kiss, 1998).

In hippocampal principal neurones, AMPA receptors desensitize relatively slowly and exhibit low Ca\(^{2+}\)-permeability (Mayer and Westbrook, 1987; Colquhoun et al., 1992; Livsey et al., 1993; Hestrin, 1993; Jonas et al., 1994). Using the reverse transcriptase-polymerase chain reaction (RT-PCR), Bochet et al., (1994) have demonstrated that GluR2 subunits are not present in a population of cultured hippocampal neurones for which the receptors show marked Ca\(^{2+}\) permeability and inward rectification.

In the CA3 region of the hippocampus only the flip version of GluR1, 2, and 3 is observed, and both flip and flop exist in the CA1 region (Sommer et al., 1990; Bochet et al., 1994; Geiger et al., 1995; Fletcher and Lodge, 1996). The expression of AMPA subtypes in the rat hippocampus is shown in table 1.1.

The schematic of an AMPA receptor (Figure 1.6) represents a cutaway view of the receptor in the Schaffer collateral pathway between CA3 and CA1 hippocampal neurones. This model includes a model of the putative topology for the individual subunits (Hollmann et al., 1994).

Cyclothiazide (CYZ) and IDRA 21 bind to a putative thiazide modulatory site to reduce AMPA receptor desensitization, with a particularly important interaction with the serine 750 (Ser750) residue within the flip-flop region.

The putative 2,3-benzodiazepine modulatory site for GYKI exerts a negative allosteric effect upon the AMPA receptor depicted by the gray arrowhead, and the effect is not necessarily dependent upon interaction with the flip-flop region.
Chapter 1

The thiazide and 2,3-benzodiazepine sites are probably distinct modulatory sites, but they allosterically oppose each other's actions, depicted by opposing arrowheads. Similarly, the benzoylpiperidine site is distinct, but Ser750 mutations influence its modulatory actions, suggesting that the flip/flop region may be important in mediating its effects.

It must be noted that AMPA responses from different neurones have different sensitivity to various modulators, and these can be mimicked in recombinant systems by changing the combinations of AMPA receptor subunit subtypes expressed, suggesting that molecular variability confers physiologically important differences that diversify the kinetics and time course of AMPA receptor-mediated synaptic responses (Fleck et al., 1996).

Table 1.1  Expression of AMPA subtypes in the rat hippocampus (Fletcher & Lodge, 1996):

<table>
<thead>
<tr>
<th>AMPA subtype</th>
<th>Dentate granule</th>
<th>CA1 pyramidal</th>
<th>CA3 pyramidal</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluR1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GluR2</td>
<td>+</td>
<td>+flip</td>
<td>+</td>
</tr>
<tr>
<td>GluR3</td>
<td>+</td>
<td>+flop</td>
<td>+flip</td>
</tr>
<tr>
<td>GluR4</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1.6  Schematic representation of the Schaffer collateral pathway between the CA3 and CA1 hippocampal neurones which is a glutamatergic excitatory pathway, (adapted and modified from Yamada, 1998). The signaling between neurones in this region is primarily mediated by AMPA receptors.
1.7 Developmental Regulation of AMPA Receptor

Early ligand binding studies demonstrated developmental changes in the expression of AMPA receptors in various brain regions (Insel et al., 1990; Miller et al., 1990). In the hippocampus, the number of binding sites were shown to increase within the first four weeks of post-natal development. In situ hybridization (Monyer et al., 1991; Pellegrini-Giampierto et al., 1992; Standley et al., 1995) and Northern blot analysis (Durand and Zukin, 1993) have confirmed that AMPA receptor subunit expression is developmentally regulated with differences in expression of AMPA receptor splice variants during ontogenesis.

The expression of the flip/flop isoform also varies regionally within the CNS. The predominant expression in the neonate of the flip isoform, at which glutamate evokes a greater maximal current, may contribute towards synaptogenesis; the flop version is however, expressed mainly in the adult CNS (Sommer at al., 1990). The gradual increase in the expression of flop versions occurs during postnatal stages until the adult complement is reached by postnatal day 14 (Monyer et al., 1991).

The expression of GluR1 and GluR2 does not seem to be developmentally regulated in the hippocampus and cerebellum compared to the basal ganglia, cortex and thalamus, which demonstrate higher levels of GluR1 and GluR2 in neonates compared to adults. GluR3 and GluR4 subunit expression is significantly lower in both neonates and adults compared to GluR1 and GluR2. GluR3 subunit expression decreases modestly in the basal ganglia, hippocampus and cerebellum with little change in cortex with age. Developmental changes in GluR4 subunit expression have been observed in the
Chapter 1

hippocampus, cerebellum and cortex, however, no differences in GluR4 expression have been observed in basal ganglia throughout development (Franciosi, 2001). This variation in AMPA receptor subunit expression with age may be implicated in the establishment and regulation of synaptogenesis (Bahr et al., 1992) and excitotoxicity (Choi, 1989; Pagliusi et al., 1994; Franciosi, 2001).

1.8 AMPA receptors in synaptic plasticity and development

Alteration in the number and/or in the properties of synaptic glutamate receptors is currently viewed as one of the plausible mechanisms for modifications of synaptic efficacy that underlie learning and memory. As well as the NMDA involvement, it has also been proposed that LTP can be due to both an increase in neurotransmitter release and to an enhanced postsynaptic AMPA/kainate response (Bliss and Collingridge, 1993; Turrigiano, 2000; Lüscher and Frerking, 2001). The catalytic subunit of protein kinase A (PKA) increases AMPA-receptor function when added to the intracellular medium in whole-cell patch-clamp recordings (Greengard et al., 1991; Wang et al., 1991). However lack of phosphorylation of AMPA receptors by PKA in vitro suggests an indirect effect through regulatory proteins (McGlade-McCulloh et al., 1993). In contrast AMPA receptors can be efficiently phosphorylated in vitro by Ca^{2+} calmodulin-dependent kinase (CaM-KII) and to a lesser extent by protein kinase C (PKC) (McGlade-McCulloh et al., 1993). Furthermore, CaM-KII enhances kainite-activated currents in cultured hippocampal neurones. These results are consistent with a role of CaMKII in increasing the postsynaptic LTD response to glutamate in synaptic plasticity (Bettler and Mulle, 1995).
Chapter 1

1.9 Mechanism of Excitotoxicity & Neuronal Cell Damage

The role of excitatory amino acids in ischemic brain injury has become well established. Excessive activation of NMDA and AMPA receptors initiates a sequence of neurochemical events which lead to neuronal cell damage and ultimately cell death (Figure 1.7) (Fujisawa et al., 1993).

Excitotoxicity in response to endogenous glutamate is manifest when the presynaptic uptake system is deficient, for instance under conditions of metabolic stress. One of the major pathways leading from glutamate receptor activation to neuronal death involves excessive Ca$^{2+}$ influx. Some neuronal populations are not affected, even at high agonist concentrations, whereas others, like CA3 hippocampal pyramidal cells or thalamic reticular neurones are very sensitive. One possible explanation for this regional selectivity, with regards to non-NMDA receptors, is that only Ca$^{2+}$-permeable AMPA receptors are involved in the excitotoxic process (Brorson et al., 1994).
Figure 1.7 Potential paths leading to neuronal injury resulting from an episode of ischaemic insult. An ischaemic episode initiates a complex pathway involving the depletion of cellular energy stores and the release of free radicals. The energy depletion permits sustained activation of glutamate receptors and the consequent entry of Ca\(^{2+}\) via NMDA receptors, (GluR2 lacking) AMPA receptors and voltage gated Ca\(^{2+}\) channels. Elevation of intracellular Ca\(^{2+}\) causes excessive activation of Ca\(^{2+}\)-dependent enzymes. Excessive neuronal depolarization results in neuronal injury and often cell death.
1.10 Steroids and neuronal modulation

Electrophysiological studies over the past decade have shown that many compounds in addition to neurotransmitters affect electrical activity in the brain. These compounds include neurosteroids synthesized in the brain as well as compounds which are released from peripheral sources such as steroids.

The classic mode of action of steroid hormones involves binding of the steroids to their respective intracellular receptors. These receptor proteins, in the presence of the hormone, then directly bind DNA in the nucleus, allowing the transcription of specific genes (Truss and Beato, 1993; Rupprecht and Holsboer, 1999).

Typically, gene transcription peaks several hours after steroid exposure, limited by the rate of protein biosynthesis (McEwen, 1991), although this latency has been reported to be as short as 7.5 minutes (Groner et al., 1983).

The view of steroid hormone behaviour solely as the modulators of gene transcription has however altered, as indicated by a plethora of experimental evidence pointing at alternative cell-signalling pathways through which steroids can regulate a wider range of functions on a broader time scale, from milliseconds to days.

Progesterone and its metabolites have a variety of diverse effects in the brain, uterus, smooth muscle, sperm and the oocyte. The effects include changes in electrophysiological excitability, induction of anaesthesia, regulation of gonadotropin secretion, regulation of oestrogen receptors, modulation of uterine contractility and induction of acrosome reaction and oocyte maturation (Mahesh et al., 1996). The latency of the effects vary from several seconds to several hours. Therefore, it is not surprising that multiple mechanisms of action are involved.
In the past decade, considerable evidence has emerged that some steroids might alter neuronal excitability via the cell surface through interaction with specific neurotransmitter receptors via non-genomic mechanisms. The term ‘neuroactive steroids’ has been adopted for steroids with these particular properties (Paul and Purdy, 1992).

A brief description of the steroid rings and the numbering sequence of the carbon atoms within their structure, which dictate the nomenclature, is described in figure 1.8.

1.10.1 Biosynthesis of neuroactive steroids

The lipophilic nature of adrenocortical, gonadal and placental hormones affords their ability to pass the blood-brain barrier. Previously, it was assumed that steroid hormones and their metabolites arose purely from these steroidogenic tissues and their action on neurotransmitter receptors was a mechanism of communication between the brain and the periphery. The discovery of aromatase (P450c19) in the brain and its role in imprinting of sexual behaviour (Naftolin et al., 1975) as well as a paper published by Baulieu and colleagues (Corpechot et al., 1981) initiated a momentum in the concept that steroids may be synthesized by the brain and nervous system, now referred to as neurosteroids. The later discovery of the presence of a range of P450 isoenzymes, many of which being metabolisers of steroid hormones (reviewed by Mensah-Nyagan et al., 1999; Compagnone and Mellon, 2000), also suggested that the brain could be making and/or accumulating its own steroids from cholesterol for some as yet unidentified purpose. Although the steroidogenic enzymes within the CNS are expressed in distinct
cell types (i.e. neurones vs. glia), the distribution does not necessarily overlap, with respect to region, as well as time in development (Mellon et al., 2001).

Investigations have revealed the presence of local regulatory control on the synthesis of neurosteroids – directly at the sites of enzyme activity (Mellon et al., 2001). It may therefore be possible to design therapeutic regimens which upregulate or downregulate the production of specific subsets of neurosteroids at their site of synthesis, subsequently altering any neuromodulatory influence.

A short depiction of the major pathways involved in the synthesis of the neurosteroids within the nervous system is shown (Figure 1.9).

Figure 1.8 The lettering of the steroid rings and numbering of the carbon atoms in the steroidal structure (showing 5β-pregnan-3α-ol-20-one (pregnanolone)). By convention, the α configuration is that lying below the general plane of the ring system and the β configurations that projecting above the plane of the ring system. The orientation of the hydrogen at C5 of the reduced pregnane (21 carbon) and androstane (19 carbon) series determines whether the A and B ring fusion is trans (5α-series) or cis (5β-series).
Figure 1.9 Outline of the synthetic pathways for neuroactive steroid synthesis in the nervous system. DHEAS, dehydroepiandrosterone sulphate; DHT, dihydrotestosterone.
1.10.2 Modulation of neurotransmitter receptors by neuroactive steroids

The modulation of neuronal excitability via an interaction with the neurotransmitter receptor, γ-aminobutyric acid type A (GABA$_A$), was first described for the steroid anaesthetic, alphaxalone (Harrison and Simmonds, 1984), followed by the 3α-reduced metabolites of progesterone and deoxycorticosterone (Majewska et al., 1986). GABA$_A$ receptors consist of various subunits that form ligand-gated ion channels with considerable homology to glycine, nicotinic acetylcholine (nAchR) and serotonin type 3 (5-HT$_3$) receptors (Paul and Purdy, 1992; Lambert et al., 1995; Wetzel et al., 1998). A variety of different classes of drugs have been shown to act through GABA$_A$ receptors such as benzodiazepines, barbiturates, clomethiazol, alcohols, anaesthetics and more recently, neuroactive steroids. The assumption of a steroid binding site at this ligand-gated ion channel is based on pharmacological studies concerning the strong stereoselectivity and structure-activity relationship of the action of neuroactive steroids at this receptor (Simmonds, 1991; Lambert et al., 1995; Covey et al., 2001). Studies using GABA$_A$/glycine receptor chimeras suggest an allostERIC action of neuroactive steroids at the N-terminal side of the middle of the second transmembrane domain of the GABA$_A$ receptor β$_1$ and/or α$_2$ subunits (Rick et al., 1998).

Using whole-cell voltage-clamp recordings, a negative modulation of 5-HT$_3$ receptors has been shown by 17α- and 17β-estradiol, testosterone and 3α- 5α-THP. Pregnenolone sulphate and cholesterol were not found to modulate these receptors (Wetzel et al., 1998; Rupprecht, 2002). Although the subunit composition of 5-HT$_3$ receptors is far less complex than that of the GABA$_A$, the 5-HT$_3$ receptors, like GABA$_A$, belong to the
family of ligand-gated ion channels with four transmembrane spanning domains (Tecott and Julius, 1993).

Table 1.2 Neurotransmitter receptors affected by neurosteroids

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Steroid</th>
<th>Type of modulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>3α5α and 3α5β derivatives of progesterone, 11 deoxycorticosterone and testosterone</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Pregnenolone sulphate and Dehydroepiandrosterone sulphate</td>
<td>Negative</td>
</tr>
<tr>
<td>NMDA</td>
<td>17β-Oestriadiol</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Pregnenolone sulphate and Dehydroepiandrosterone sulphate</td>
<td>Positive</td>
</tr>
<tr>
<td>AMPA</td>
<td>Pregnenolone sulphate</td>
<td>Negative</td>
</tr>
<tr>
<td>Kainate</td>
<td>17β-Oestriadiol and progesterone</td>
<td>Positive</td>
</tr>
<tr>
<td>Glycine</td>
<td>Progesterone and Pregnenolone sulphate</td>
<td>Negative</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;3&lt;/sub&gt;</td>
<td>(17α- and 17β-oestradiol, progesterone, testosterone and 3α5α tetrahydroprogesterone</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Pregnenolone sulphate</td>
<td>No effect</td>
</tr>
<tr>
<td>Sigma type I</td>
<td>Dehydroepiandrosterone sulphate</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Pregnenolone sulphate</td>
<td>Negative</td>
</tr>
<tr>
<td>nAChR</td>
<td>Progesterone</td>
<td>Negative</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Progesterone</td>
<td>Negative</td>
</tr>
</tbody>
</table>

So far, other members within the family of ligand-gated ion channels (Table 1.2), e.g. nicotinic acetylcholine receptors (Valera et al., 1992; Bullock et al., 1997) or glycine receptors (Wu et al., 1990; Maksay et al., 2001) have been shown to be steroid sensitive. Again, the structure-activity requirements for the modulation of ligand-gated ion channels by steroids apparently differ considerably between members of these neurotransmitter families. The oxytocin receptor was recently identified as the first G-protein coupled receptor for which steroids can be ligands (Grazzini et al., 1998). Also
sigma receptors, which yet lack detailed molecular characterization, may bind steroids (Su et al., 1988) and are sensitive to steroid modulation (Monnet et al., 1995; Maurice et al., 2001).

Within the glutamate receptor family, NMDA (Bowlby, 1993; Bergeron et al., 1996; Gasior et al., 1997; Park-Chung et al., 1997; Yaghoubi et al., 1998; Cyr et al., 2001; Ceccon et al., 2001; Shirakawa, 2002) as well as AMPA (Bowlby, 1993; Park-Chung et al., 1994; Richardson and Wakerley, 1998; Yaghoubi et al., 1998; Rupprecht and Holsboer, 1999; Cyr et al., 2001) and kainate (Park-Chung et al., 1994; Wu et al., 1998) receptors have also been demonstrated to be targets for steroid modulation. Alterations in the concentration of distinct steroids in the brain can therefore affect multiple neurotransmitter receptors with a plethora of effects on neuronal excitability.

1.10.3 Modulation of AMPA receptors by neuroactive steroids

In cerebellar purkinje cells, systemic administration of progesterone (50μg, i.v.) has been reported to attenuate neuronal responses to iontophoretically applied AMPA by 76% within minutes (Smith, 1991).

Studies examining the effect of pregnenolone sulphate on activity of vasopressin neurones, has been shown to result in depressed firing in the presence of AMPA within 5 minutes of perfusion (Richardson and Wakerley, 1998). Whole-cell patch clamp studies, using hippocampal cultured neurones (Bowlby, 1993), also caused a reduction in the evoked AMPA currents by this neurosteroid.
At the GluR1 and GluR3 receptor subtypes, pregnenolone sulphate has been shown to reduce the evoked currents, with a noncompetitive mechanism of inhibition (Yaghoubi et al., 1998). Pregnenolone hemisuccinate is a more selective modulator of AMPA receptors, compared to pregnenolone sulphate, inhibiting GluR1 and GluR3 responses by over 70%, while reducing the GluR6 response by only 15% (Yaghoubi et al., 1998), suggesting that it may be possible to develop selective steroids that specifically target particular AMPA subtypes.
Chapter 2

Materials and Methods
Chapter 2

2.1 Introduction

In order to pursue the aims of this project, biochemical, electrophysiological and analytical techniques were employed. A suspension of acutely dissociated neurones was first obtained. This was followed by a membrane cholesterol modulation process. The neurone suspensions were later assayed for cholesterol and protein content, subsequent to the removal of samples of the pretreated neurones for electrophysiological recordings.

2.2 Dissociation of Hippocampal Neurones

Hippocampal neurones were obtained from male Wistar rats aged 12-16 days post natal by a method adapted from Sooksawate & Simmonds (1998). Following cervical dislocation and decapitation, the brain was rapidly removed and placed in ice-cold oxygenated buffer containing (mM): NaCl 150, KCl 3, CaCl₂ 1.2, MgCl₂ 1.8, glucose 11, HEPES 10 (pH 7.35). The areas containing the hippocampus were then sliced into 400μm thick sections using a Vibroslice (Campden Instruments, UK). Subsequent to an approximate 20-30 minute preincubation in oxygen-saturated buffer at room temperature, the slices were enzymatically treated by incubation in buffer containing 0.04% (w/v) protease type XIV (pronase), and then in 0.04% (w/v) protease type X (thermolysin) for 20 minutes each at 31°C. The slices were washed five times with buffer to terminate the enzyme treatment.

The hippocampal region was then separated and the slices were gently triturated using fire-polished glass pipettes of decreasing tip sizes of 2, 1 and 0.5mm respectively. The resulting neurone suspension was then allowed to stand for 10 minutes before removing the supernatant and repeating the dissociation with the remaining suspension containing
tissue aggregates. The suspension was then centrifuged at 175 xg (Centaur 2E, MSE, UK) for 3 minutes. The loose pellet of neurones was resuspended in fresh buffer and layered onto a 5% (w/v) solution of bovine serum albumin (BSA) in buffer for further centrifugation as previously to separate and isolate the neurones from suspended cell debris. The neurones were finally resuspended and centrifuged as before to remove any traces of BSA.

The acutely dissociated cells consequently obtained were suitable for electrophysiological recording after being allowed to adhere to the base of the recording chamber for 40-50 minutes.

Figure 2.1 True phase photomicrograph of an acutely dissociated hippocampal neurone, prior to cholesterol manipulation procedure. Scale bar, 30μm.
2.3 Preparation of methyl-β-cyclodextrin solution and the cholesterol inclusion complex

Methyl-β-cyclodextrin was dissolved in buffer at a concentration of 50mM. The stock solution was stored as 500μl aliquots at -20°C.

The cholesterol-methyl-β-cyclodextrin complex was prepared by adding cholesterol to 15mM methyl-β-cyclodextrin in buffer to make a concentration of 1.5mM. The mixture was overlaid with nitrogen in an airtight glass container and stirred at room temperature for about 6 hours for successful dissolution of cholesterol into the complex. The complex was subsequently filtered through a 0.22μm Millipore filter (Millipore, UK). The stock solution was then divided into 500μl aliquots, and individually overlaid with nitrogen before storage at -20°C.

The frozen aliquots of methyl-β-cyclodextrin or the cholesterol inclusion complex were thawed prior to the incubations and added to the neurone suspensions to make incubation concentrations of 5mM and 1.5 mM methyl-β-cyclodextrin for membrane cholesterol depletion and enrichment, respectively.

2.4 Preparation of epicholesterol-methyl-β-cyclodextrin inclusion complex

This enrichment vehicle was prepared using the same procedure as cholesterol-methyl-β-cyclodextrin complex. The epicholesterol inclusion mixture was however stirred for about 24 hours for the complete dissolution of the sterol.
2.5 Manipulation of membrane cholesterol using methyl-β-cyclodextrin and its cholesterol and epicholesterol inclusion complexes

1) Cholesterol Depletion
The neurone suspensions were incubated in oxygenated buffer solution containing 5mM methyl-β-cyclodextrin at 31°C for 20-30 minutes. The length of incubation was dependent on the degree of membrane cholesterol reduction required. The depletion was terminated by centrifugation at 340 xg for 3 minutes and two washes of the neurones with buffer.

2) Cholesterol Enrichment
The neurone suspensions were incubated in oxygenated buffer containing cholesterol-methyl-β-cyclodextrin complex at a concentration of 0.15mM cholesterol under the same conditions as described in cholesterol depletion. For the control group, the neurone suspensions were subjected to the same procedure without the modulating complex.

3) Restoration of membrane cholesterol following depletion
The samples of cholesterol depleted neurones were incubated in oxygenated buffer containing cholesterol-methyl-β-cyclodextrin complex at 0.15mM cholesterol at 31°C for 5-20 minutes. The cholesterol transfer was terminated by centrifugation at 340 xg for 3 minutes and two washes of the neurones with buffer.
4) Epicholesterol enrichment

The neurone suspension was incubated in oxygenated buffer containing epicholesterol-methyl-β-cyclodextrin complex at a final concentration of 0.15mM epicholesterol at 31°C for 30 minutes. The transfer of epicholesterol was terminated by centrifugation at 340 xg for 3 minutes and two washes of the neurone suspension with buffer.

5) Incubation of cholesterol-depleted neurones with epicholesterol

The depleted neurones were incubated in oxygenated buffer containing epicholesterol-methyl-β-cyclodextrin complex at 0.15mM epicholesterol at 31°C for 30 minutes. The transfer of epicholesterol was terminated by centrifugation at 340 xg for 3 minutes and two washes of the neurones with buffer.

2.6 Cholesterol and protein assays of hippocampal neurones

Following the removal of samples of the pretreated neurones for electrophysiological recording, the neurone suspensions were centrifuged at 340 xg for 3 minutes and resuspended in wash buffer containing (mM): Tris-base 5, EDTA 1, pH 7.4 at 4°C. The samples were then stored at –20°C for later assays of cholesterol and protein.

The thawed neurone suspensions were homogenised (Ultra Turrax Homogeniser, Germany) for carrying out the assays, using ice-cold wash buffer. The samples were
then centrifuged at 48,400 x g (J2-21M/E, BECKMAN, UK), and resuspended in wash buffer.

Cholesterol assays were carried out on the samples using the Sigma INFINITY cholesterol diagnostic kit which measured total cholesterol enzymatically and was modified from the method of Allain et al., (1974).

The method is based on the hydrolysis of cholesterol esters to cholesterol by the enzyme cholesterol esterase. The free cholesterol was then oxidised by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide was then coupled with the chromogen, 4-aminoantipyrine and p-hydroxybenzenesulfonate in the presence of peroxidase to yield a quinoneimine dye with an absorbance maximum of 500nm. The calibration curve for cholesterol assays is shown in figure 2.2. Cholesterol calibrator (Sigma, UK) was used as the standard cholesterol.

The calibration for the tissue proteins levels was significantly modified from methods described by Sooksawate and Simmonds (1998), in order to fully homogenise the protein fractions in the samples.

Because the sensitivity of the assay depends on the nature of the protein, complex samples, in which the proteins differ widely in their response to the assay can be difficult to quantitate. But modifications such as the addition of a subthreshold amount of sodium dodecyl sulphate (SDS), has been shown to improve the effectiveness of the Bio-Rad Protein Assay.

SDS is an anionic surfactant which is widely used in electrophoretic separation and molecular weight determination of proteins. In this investigation 0.1%w/v concentration
of SDS showed the highest level of tissue protein solubilisation, with the least amount of deviation from the Beer-Lambert law. The linearity of the calibration line can be affected by such forces as hydrogen bonding and Van der Waals forces between the interacting sample molecules.

Protein assays were therefore carried out on the neurone suspensions incorporating 0.1\%w/v SDS, using the BioRad Detergent Compatible Protein Assay (BioRad, UK). The assay was based on the shift of the absorbance maximum from 465nm to 595nm of Coomassie brilliant blue G-250 dye in response to concentrations of protein (Bradford, 1976). The calibration curve for protein assays is shown in figure 2.3. Lipholised protein calibrator (BioRad, UK) was used as the standard protein.
Figure 2.2  Standard curve of cholesterol measured by cholesterol diagnostic kit (Sigma, US). Cholesterol calibrator (Sigma, USA) was used as standard cholesterol. All data points are the means of triplicates.

Figure 2.3  Standard curve of protein measured by BioRad Detergent Compatible Protein Assay kit from BIO-RAD (Germany). Lyophilized protein standard BIO-RAD (Germany) was used as standard protein. All data points are the means of triplicates.
2.7 Electrophysiological measurements

Electrophysiological recordings were performed in an earthed Faraday cage to reduce interference from external electrical sources and on an anti-vibration air-table to reduce physical interference.

The patch clamp technique for whole-cell recording (Hamil et al., 1981) was used to study the effects of membrane cholesterol and neurosteroids on the AMPA receptors of acutely dissociated hippocampal neurones. Patch pipettes were pulled from thin-walled borosilicate microelectrode glass (GC-150-T10) (Harvard Apparatus, UK) using a two-stage vertical puller (L/M-3P-A, List-Medical, Germany). The pipettes were then filled with an internal solution containing (mM): CsCl 140, CaCl$_2$ 0.5, MgCl$_2$ 2, Na$_2$ATP 2, EGTA 10 and HEPES 10 (pH 7.2).

Whole cell membrane currents were recorded with patch pipettes of 2-5 MΩ resistance and the membrane voltage clamped at -40mV. During recording, neurones were superfused with buffer at a rate of 1-2 ml/min. Membrane current was measured using a patch clamp amplifier (EPC-7, List-Electronic, Germany) and was monitored simultaneously on an oscillioscope (GOULD, UK) and a pen recorder (GOULD, France). All experiments were performed at room temperature (20°C). Additional experiments were also carried out using AxoClamp-2A amplifier with a DigiData 1200 interface (Axon Instruments, Inc., US), pClamp 6.0 software (Axon), and an IBM-compatible Pentium-90 PC (Viglen), upon availability of the equipment at latter stages. Rapid drug applications were carried out using fabricated glass U-tubes which permitted effective and simultaneous activation of the receptor channels in whole cell recordings.
2.8 Construction of a U-tube

The U-tube was moulded using standard borosilicate microelectrode glass (GC-150-T10) (Harvard Apparatus, UK). A single stage pull was performed on the glass (Figure 2.4A). The U-shape was formed over the pilot light of a bunsen burner by gently heating the extruded neck and allowing one side to fall forming an angle of approximately 30° (Figure 2.4B). The U-tube structure was reinforced by attachment of smaller rods of borosilicate glass along its length using Araldite resin (Figure 2.4C) and allowed to cure over night.

Sealing one end of the U-tube, the drug injection port was created by gently applying pressure to the open end using a 2ml syringe and silicone tubing, while exposing the apex of the glass to the micro-burner (Figure 2.4D). The hole was then strengthened and reduced to an appropriate size by polishing in gradual stages using a continuously glowing element, while monitoring the progress under the microscope (50 x magnification). A length of microelectrode glass was also used as a separate wash-off tube. First the glass was fire polished until the bore was reduced to about half of the original diameter. Then using the micro-burner, the tip of the glass was shaped so as to follow the contours of the apex of the U-tube. The wash-off tube was then glued in place such that the tip was placed a few millimeters back from the U-tube apex, and that the outflows from both crossed over as close to the U-tube apex as possible (Figure 2.4E).

The U-tube was then connected to a perfusion system which incorporated controlling two-way silastic solenoid valves (12V) (The Lee Co., UK) as outlined (Figure 2.4F). The system allowed the flexibility of application of different drug concentrations for the
Chapter 2

generation of a concentration-response curve and also quick administration through either the pre-incubation (wash tube) or direct application (U-tube). Prior to use, the suitability of the U-tube and correct directional flow of the injection port was tested using a potassium permanganate solution, viewed under the microscope.

The tip of the U-tube was placed approximately 300μm from the neurone to be recorded. The reservoirs of drug solutions were placed at about 30cm above the level of the U-tube. The system was connected to a vacuum pump (HY-Flo, Medcalf Bros., UK). While in the non-injection mode, the U-tube also facilitated the exchange of solution within the recording chamber. The system allowed for consistent 1.0 second applications of increasing concentrations of AMPA (0.1-300μM), in the absence and thereafter in combination with the modulator to the same neurone.
Figure 2.4  Schematic diagram showing the stages of U-tube fabrication process
2.9 Standardization of AMPA current responses

Brief applications of AMPA (1.0 second) to voltage clamped dissociated hippocampal neurones (-40mV), resulted in fast activating inward currents which characteristically displayed very different desensitization patterns from cell to cell.

The receptor response to AMPA was typically found to consist of two components (Figure 2.5), a fast peak amplitude and a steady-state response. The variations amongst native hippocampal neurones with regards to desensitization behaviour, possibly due to different GluR subunit compositions (refer to Chapter 1), were minimized by selective exclusion of atypical responses.

About 80% of the cells were found to exhibit a rapid desensitization to reach a plateau level, while others desensitized almost completely, and some did not desensitize at all. In order to standardize the receptor responses, the latter two types of cells were excluded from the experiments.

Figure 2.5 Schematic diagram showing AMPA receptor response to a 1.0 second application of AMPA in comparison to GABA receptor response to GABA.
It was decided that the peak and plateau phase responses should be investigated independently so that any possible influence on either component of the response by cholesterol or a modulator could be better investigated. The data were therefore expressed as a normalization to the projected maximum response for both peak and plateau components of AMPA responses.

2.10 Drugs and chemicals

AMPA was freshly dissolved in buffer at a concentration of 10mM on the day of each experiment. Cholesterol for acute application was dissolved in acetone at a concentration of 40mM, and mixed with dimethyl sulfoxide (DMSO) to achieve a concentration of 10mM and diluted to 1μM in buffer. The final concentrations of acetone and DMSO were less than 0.0025 and 0.01% (v/v), respectively. All neurosteroids were dissolved in DMSO at a concentration of 20mM. NBQX, GYKI52466 and picrotoxinin were dissolved in DMSO at a concentration of 10mM. All drug stock solutions were then diluted to appropriate concentrations in buffer. The final concentration of DMSO was less than 0.1% (v/v) for all drug solutions. The same concentration of DMSO was added to all control AMPA solutions to maintain consistency.

All drugs and chemicals were purchased from Sigma (USA) with the exception of the BioRad Detergent Compatible Protein Assay from BIO-RAD (Germany), methyl-β-cyclodextrin from Aldrich (UK), AMPA, NBQX and GYKI52466 from Tocris (Bristol, UK), CsCl, MgCl₂, CaCl₂ and acetone from BDH (Poole, UK). Epicholesterol was purchased from Steraloids inc. (USA).
2.11 Data Analysis

For concentration-response analysis, data from each neurone were plotted using GraphPad PRISM™ (GraphPad Software, USA) and fitted with a logistic equation in the form:

\[ I = I_{\text{min}} + \left( \frac{(I_{\text{max}} - I_{\text{min}})}{EC_{50}([X])} \right)^H \]

where \( I \) is the AMPA current at AMPA concentration \([X]\). \( I_{\text{min}} \) and \( I_{\text{max}} \) are the minimal and the maximal responses, \( EC_{50} \) is the concentration of AMPA eliciting 50\% of the maximal response and \( H \) is the Hill coefficient. Peak amplitudes were measured at the absolute maximum of the currents, taking into account the noise of the baseline. The amplitudes of steady-state currents were measured at 250ms from the start of agonist application.

Upon the availability of electronic means of data capture, the decay phases of the recorded currents evoked by AMPA, were fitted by a single exponential according to the Chebychev equation:

\[ F(t) = \sum_{i=1}^{n} A_i e^{-\tau_i t} + C \]

solving for the amplitude \( A \), the time constant \( \tau \), and the constant \( y \)-offset \( C \) for each component \( i \).

For statistical analysis, a ratio of \( EC_{50} \) values for AMPA in the presence and absence of various compounds was determined from each experiment.

Combined data are expressed as mean±standard error of the mean. Significance was tested by ANOVA using the Dunnett method of analysis or Student’s \( t \)-test, where applicable, and a \( p \) value of less than 0.05 was considered to be significant.
2.12 Discussion

The dissociated cells plated for electrophysiological recordings were about 20-50μm in diameter, with a cell density of approximately 150-200 neurones. For the purpose of consistency, the cells within an approximate size of 30μm were selected for recordings. Some neurones possessed one or more processes, while others only consisted of the somata, with the processes possibly being lost during the membrane cholesterol manipulation stages. These cells were actually preferred to the former for whole cell recordings as clamping the holding potential is generally facilitated by a circular morphology.

There has been an extensive use of cyclodextrins or their derivatives as drug delivery vehicles (Pitha et al., 1988). They are water-soluble cyclic oligomers of glucose with a hydrophobic cavity capable of forming inclusion complexes to enhance the solubility of nonpolar substances. The selective extraction of cholesterol from plasma membranes by β-cyclodextrin in preference to other membrane lipids has been described by Ohtani et al., (1989). Methyl-β-cyclodextrin has been shown to be a more selective substance compared to other β-cyclodextrins (β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin in depleting cholesterol from cultured cells (Kilsdonk et al., 1995). The incubation protocols with methyl-β-cyclodextrin described in this Chapter provided high and low levels of cholesterol enrichment and depletion of neurones described in Chapter 3.

Considering that the plasma membrane of a cell contains more than 90% of the cholesterol present within that cell (Yeagle, 1993), it has been presumed that the extra
cholesterol accumulated during the cholesterol enrichment procedure was located predominantly in the plasma membrane. For the cholesterol depletion procedure, it has also been presumed that cholesterol was transferred from the plasma membrane to the acceptor, methyl-β-cyclodextrin.

The advantage of using methyl-β-cyclodextrin and its cholesterol inclusion complex is that the membrane cholesterol of membrane fragments and cultured cells can be reversibly changed (Gimple et al., 1997). This was exploited in the present investigations where initial cholesterol depletion by incubation with methyl-β-cyclodextrin could be restored to control levels by incubation with cholesterol-methyl-β-cyclodextrin complex. Such a procedure allowed discrimination between possible cholesterol-dependent changes in neuronal properties and changes due to some effects of methyl-β-cyclodextrin.

The changes in membrane cholesterol reported here with methyl-β-cyclodextrin as a cholesterol carrier were sufficient for the purposes of this study but more extreme changes were found to be incompatible with satisfactory electrophysiological properties and neuronal viability.

With regards to the statistical analysis, the Dunnett method assumed that the scatter of data followed a normal distribution. This method provided various statistical post-tests and offered the ability to carry out multiple comparisons of data (i.e. each data set could be compared against other data columns, as well as the control values). Another advantage of the Dunnett method was that it permitted the use of data with different sample numbers.
Chapter 3

Manipulation of Membrane Cholesterol
3.1 Introduction

The methods commonly employed to alter lipid composition of isolated plasma membranes or living cells include the incubation with liposomes of defined composition (Klein et al., 1978; Whetton et. al., 1983; Bennett and Simmonds, 1996; Sooksawate and Simmonds, 1998), the use of non-specific lipid transfer proteins (Castuma and Brenner, 1986), the preparation of highly diluted aqueous cholesterol solutions (Crews et al., 1983), the use of cholesterol oxidase (Gimple et. al., 1997), and the use of cyclodextrin and its cholesterol inclusion complex (Kilsdonk et. al., 1995; Gimple et. al., 1997; Sooksawate and Simmonds, 1998, 2001). An advantage of the use of cyclodextrins over liposomal preparations was a far more rapid cholesterol transfer process and hence shorter incubation times of neurones with methyl-β-cyclodextrin (Sooksawate and Simmonds, 1998). Incubations with methyl-β-cyclodextrin and its cholesterol inclusion complex, carried out previously in this laboratory were utilized in the present study with some modifications.

Cyclodextrins, which are cyclical oligomers of glucose, have the capacity to sequester lipophiles in their hydrophobic core and form inclusion complexes with nonpolar substances. They have a potentially broad spectrum of research applications and are widely used in drug formulation (Stella and Rajewski, 1997; Khomutov et al., 2002). The substituted β-cyclodextrins are known to form soluble inclusion complexes with cholesterol and are also able to provide an additional pool of cholesterol in the aqueous phase (Pitha and Pitha, 1985). This made such a cholesterol transfer vehicle a suitable choice for the current project, as it also provided the possibility of membrane cholesterol restoration.
3.2 The viability of acutely dissociated hippocampal neurones

Following the dissociation protocol, about 90% of the hippocampal neurones were viable (trypan blue exclusion). The neurones remaining viable after 1 hour incubation with 5mM methyl-β-cyclodextrin or 0.15mM cholesterol + 1.5mM methyl-β-cyclodextrin inclusion complex were 70-90%. Most of the acutely dissociated neurones had lost or resorbed their processes following the cholesterol manipulation procedures. This included the control neurones which had been treated in the same manner by incubation for 30 minutes at 31°C in buffer. Less than 50% of the neurones were still viable after plating into a recording chamber for 4 hours.

3.3 Alteration of membrane cholesterol content by methyl-β-cyclodextrin and its cholesterol inclusion complex

Samples of the acutely dissociated neurones were incubated in different concentrations of the depletion and enrichment vehicle for various lengths of time in order to assess the degree of membrane cholesterol alteration achieved. After incubation with cholesterol-methyl-β-cyclodextrin at a final concentration of 0.15 or 0.3mM cholesterol for 5-30 minutes, cholesterol content of the neurones could be enriched to within the range 133±1.5% to 244±2.7% of control neurones. Incubation with methyl-β-cyclodextrin at a final concentration of 5 or 10mM 5-30 minutes, resulted in membrane cholesterol depletion to within the range 80±1.4% to 59±1.9% of control neurones respectively (Figure 3.1 and Table 3.1)
Table 3.1  Alterations of membrane cholesterol by methyl-β-cyclodextrin in acutely dissociated hippocampal neurones. Membrane cholesterol was manipulated by incubation at 31°C for 5-30 minutes with 5 and 10mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Chol-MβC) at a final concentration of 0.15 and 0.30mM cholesterol (enrichment). For details of method refer to Chapter 2.

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Cholesterol content of neurones (μmole.mg protein⁻¹)</th>
<th>Cholesterol content of neurones (μmole.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enrichment [Chol-MβC]</td>
<td>Depletion [MβC]</td>
</tr>
<tr>
<td></td>
<td>0.15mM Chol + 1.5mM MβC</td>
<td>0.3mM Chol + 3mM MβC</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.397±0.013 (5)</td>
<td>0.440±0.008(4)</td>
<td>0.479±0.009(4)</td>
</tr>
<tr>
<td>5</td>
<td>0.533±0.021* (3)</td>
<td>0.804±0.045**(3)</td>
</tr>
<tr>
<td>10</td>
<td>0.769±0.040** (6)</td>
<td>0.938±0.039** (5)</td>
</tr>
<tr>
<td>20</td>
<td>0.876±0.070** (6)</td>
<td>1.073±0.028** (4)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of experiments are shown in parenthesis.
ND=not done; NS=not significant
*P<0.05 (from control; two-way ANOVA), **P<0.01
Figure 3.1  Alteration of membrane cholesterol by methyl-β-cyclodextrin in acutely dissociated hippocampal neurones. Membrane cholesterol was manipulated by incubation with 5 and 10mM methyl-β-cyclodextrin (depletion) or 0.15 and 0.3mM cholesterol complexed with a 10-fold molar excess of methyl-β-cyclodextrin (cholesterol enrichment) at 31°C for 5-30 minutes (n=3-6). (▲) Enrichment (0.3mM Cholesterol + 3mM Mβc), (■) Enrichment (0.15mM Chol + 1.5mM Mβc), (■) Depletion (5mM Mβc) and (▼) Depletion (10mM Mβc).
Although the use of 10mM methyl-β-cyclodextrin and 0.3mM cholesterol-methyl-β-cyclodextrin complex were found to result in the highest degree of depletion and enrichment, respectively, the difficulty of obtaining stable whole cell patch clamp recordings, and also an apparent reduction of cell viability with the passage of time, did not make such neurones suitable for electrophysiological investigations. The subsequent whole cell patch clamp recordings utilizing these cells were also found to give rise to AMPA response $EC_{50}$s with a large standard deviation from the mean (data not shown).

Upon analysis of the incubation protocols together with the corresponding AMPA $EC_{50}$ values of the normalized responses it was decided that the protocol for all subsequent membrane cholesterol manipulations must be standardized so as to afford maximal alteration of membrane cholesterol which resulted in most reproducible $EC_{50}$ values. The cholesterol depletion and enrichments were subsequently carried out by incubation with 5mM methyl-β-cyclodextrin and 0.15mM cholesterol inclusion complex, respectively, for up to 30 minutes and were found to be satisfactory with patch clamp recordings (refer to cholesterol manipulation protocol in Chapter 2).
3.4 Effect of time elapsed on membrane cholesterol content of neurones

Since membrane cholesterol levels were routinely measured immediately after the incubation procedures, the question was raised of whether these levels fairly reflect the condition of the single neurones at the time of recording.

Table 3.2 Time elapsed following the incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol (enrichment).

<table>
<thead>
<tr>
<th>Time elapsed (hrs)</th>
<th>Cholesterol content of neurones (μmole.mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enrichment [Chol-MβC]</td>
</tr>
<tr>
<td>Control</td>
<td>0.404±0.008 (n=6)</td>
</tr>
<tr>
<td>Incubation (30 mins)</td>
<td>0.879±0.057 (n=3)</td>
</tr>
<tr>
<td>After 2 hrs</td>
<td>0.881±0.023 (n=5)</td>
</tr>
<tr>
<td>After 5 hrs</td>
<td>0.873±0.014 (n=6)</td>
</tr>
</tbody>
</table>

The cholesterol content of neurones for enriched and depleted neurones (Table 3.2) was found to remain stable ($P>0.05$; two-way ANOVA) during the time-course of the electrophysiological recordings.
3.5 Discussion

Despite extensive investigation, the principal mechanisms by which cholesterol is transferred between donor and acceptor membranes are not fully established (Bittman, 1993). From the fact that the plasma membrane of a cell contains more than 90% of the cholesterol present within that cell (Yeagle, 1993), it has been presumed that the extra cholesterol accumulated during the cholesterol enrichment procedure was located predominantly in the plasma membrane. For the cholesterol depletion procedure, it has been presumed that cholesterol was transferred from the plasma membrane to the acceptor of cholesterol, methyl-β-cyclodextrin.

One advantage of using methyl-β-cyclodextrin and its cholesterol inclusion complex is that the membrane cholesterol of membrane fragments and cultured cells can be reversibly changed (Gimpl et al., 1997). This property was later exploited where initial cholesterol depletion was followed by incubation with the cholesterol inclusion complex.

It was also important to investigate the possibility of any changes in the level of membrane cholesterol depletion or enrichment throughout the time course of the corresponding electrophysiological recordings. The homeostasis of plasma membrane is crucial to cell survival in vivo and neurones expend much energy to ensure that the plasma cholesterol content is optimal for cell function (Yeagle, 1991). The very small amount of tissue plated out for whole-cell patch clamp recordings was insufficient for a cholesterol assay to be performed at the end of the experiments. It was therefore decided to measure the cholesterol content of a number of batches of enriched and depleted
neurones at different time intervals following the incubations (Table 3.2). The cholesterol content of neurones for enriched and depleted neurones were not found to alter during the time interval of the whole-cell recordings. Although the glial influence in cholesterol synthesis/turnover would be absent for acutely dissociated neurones, such cells may yet have the capacity to synthesize cholesterol to overcome a deficit (i.e. a depleted state). But it must be noted that the rate of cholesterol turnover is perhaps too slow to influence this type of investigation.

The subsequent alterations of AMPA EC$_{50}$ values caused by both cholesterol depletion and enrichment have also been highly reproducible despite their being obtained from neurones over a wide time range, from 50 minutes to 5-6 hours after plating.
Chapter 4

Effect of Membrane Cholesterol on the Sensitivity of AMPA Receptors to AMPA
4.1 Introduction

The role of cholesterol as an essential structural element of cellular membranes is well recognized, but it is becoming apparent that membrane lipids can also influence cellular function through interaction with specific recognition sites on some membrane proteins (Yeagle, 1993). As a result, when investigating the role of cholesterol, much research has been specifically focused on the nature of the lipid-protein interactions.

While taking the cholesterol influence on overall mobility of membrane proteins into account, no specific cholesterol-receptor interactions have been found in studies involving the 5-HT3 (Wetzel et al., 1998) and cholecystokinin receptors (Gimpl et al., 1997). However, a mechanism of direct modulation has been proposed for several other receptors such as the oxytocin receptor (Gimpl et al., 1997), nicotinic acetylcholine receptor (Narayanaswami & McNamee, 1993; Fernandez-Ballester, et al., 1994; Addona et al., 1998), rhodopsin (Albert et al., 1996) and also the GABA\textsubscript{A} receptor (Sooksawate & Simmonds, 1998). Whether cholesterol plays a specific role in the functioning of many other integral membrane receptors has yet to be determined.

The objective of this study was an analogous investigation of the role of membrane cholesterol on the function of the AMPA receptors.
4.2 Effect of membrane cholesterol on AMPA currents

Examples of the currents induced by increasing the concentration of AMPA on control, cholesterol-depleted and enriched neurones are shown in figure 4.1. The AMPA currents in the enriched neurones can be seen to be lower at every concentration compared to those of the control. Cholesterol depletion, however, resulted in larger induced currents. There are also apparent differences in the peak/plateau ratio of the AMPA currents. Although these examples have been selected as reasonably typical, it must be noted that the absolute currents induced and the fade to plateau in the continued presence of the agonist showed variation between individual neurones possibly due to cell-to-cell difference in size, the number of AMPA receptors, expression of various receptor subunits and splice variants. Therefore, there was a need to replicate the experiments and apply dose-response curve analysis to determine the effects of cholesterol. The cholesterol contents of the neurones used in this part of the study are shown in Table 4.1.

Table 4.1 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 1.5mM MβC (enrichment).

<table>
<thead>
<tr>
<th>Cholesterol content</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmole.mg protein⁻¹</td>
<td>0.379±0.013</td>
<td>0.782±0.058</td>
<td>0.233±0.045</td>
</tr>
<tr>
<td>Sample number</td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>206±4.8%</td>
<td>62±2.3%</td>
</tr>
</tbody>
</table>
Figure 4.1 The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on AMPA currents in acutely dissociated hippocampal neurones. (A) depleted neurone (59% of control), (B) Control neurone, (C) enriched neurone (203% of control).
4.2.1 Effect of membrane cholesterol on peak AMPA currents

At maximum agonist concentration (300-500µM), there was not a large apparent difference in the peak responses between the depleted neurones and those of the control batches, suggesting that a saturation of response level may have been reached. The enriched neurones however, exhibited a lower absolute current at maximum AMPA concentrations (Figure 4.2).

The maximal response for depleted neurones (2744±132.9 pA; n=16) was not found to be statistically significant from those of control (2568±89.3 pA; n=18). Conversely, the maximal response for enriched neurones (1502±108.5 pA; n=19) was considerably lower compared to control values (p<0.01; two-way ANOVA).

In order to compare the sensitivities to AMPA at each cholesterol state, the AMPA currents were normalized with respect to the projected maximum of the curve. This permitted comparisons of the AMPA EC<sub>50</sub> values, as well as yielding information on the Hill slope of the dose-response curves.

In repeated experiments, cholesterol enrichment was associated with an increase in the peak current EC<sub>50</sub> value (Table 4.2), and an elevated Hill slope (Figure 4.3). Cholesterol depletion, however, resulted in a decrease in peak current EC<sub>50</sub> of the acutely dissociated hippocampal neurones (Figure 4.4) and a reduced Hill slope.
Figure 4.2 AMPA dose-response relationships showing the whole cell peak current responses to AMPA recorded from acutely dissociated hippocampal neurones voltage clamped at -40mV. Error bars represent standard error of mean.

Table 4.2 The effect of membrane cholesterol alterations on peak AMPA dose-response relationships of acutely dissociated neurones. Numbers of experiments are shown in parentheses.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of neurones</td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>EC50</td>
<td>24.08±0.56 µM</td>
<td>57.5±1.02 µM</td>
<td>9.8±0.43 µM</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.39±0.04</td>
<td>1.85±0.07</td>
<td>1.02±0.06</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 4.3  AMPA dose-response relationships showing whole cell peak current responses to AMPA recorded from cholesterol-enriched acutely dissociated hippocampal neurones clamped at -40mV.

Figure 4.4  Whole cell plateau current responses to AMPA recorded from cholesterol-depleted acutely dissociated hippocampal neurones clamped at -40mV.
4.2.2 Effect of membrane cholesterol on plateau AMPA currents

The fast peak current responses to AMPA rapidly declined to produce a steady-state current upon continued exposure (1.0 second) to the agonist.

In replicate experiments, cholesterol-depleted neurones exhibited larger absolute steady-state currents compared to control neurones at every agonist concentration (Figure 4.5). This was in contrast to the effect of cholesterol enrichment which resulted in the attenuation of plateau responses to AMPA.

The elevated plateau maximal response for depleted neurones (525±40.8 pA) was found to be significantly different from those of control (393±21.7 pA). Also, the maximum current for enriched neurones (206±18.4 pA) was considerably lower compared to the control (p<0.01; two-way ANOVA).

The normalised dose-response curve for depleted neurones (Figure 4.6a) showed an increase in the EC\textsubscript{50} compared to control (Table 4.3). Cholesterol enrichment, however, resulted in a decrease of the EC\textsubscript{50} value which was also found to be statistically significant (Table 4.3).
Figure 4.5  AMPA dose-response relationships showing whole cell plateau current responses to AMPA recorded from cholesterol-enriched acutely dissociated hippocampal neurones clamped at -40mV. (▼) Depleted, (■) control and (▲) enriched neurones.

Table 4.3  The effect of membrane cholesterol alterations on plateau AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of neurones</td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>EC\textsubscript{50}</td>
<td>2.73±0.13 μM</td>
<td>1.79±0.16 μM</td>
<td>5.04±0.21 μM</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.95±0.04</td>
<td>0.78±0.07</td>
<td>1.19±0.06</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
Figure 4.6 Whole cell plateau current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV. A) (■) Control neurones (n=19), B) (▲) cholesterol-enriched neurones (n=17) and C) (▼) depleted neurones (n=13). Dotted lines in B and C represent the control curve.
4.2.3 Effect of cholesterol on the decay from peak to plateau AMPA currents

An alternative analysis of the plateau response is the measurement of the current decay from peak ($I_p$) to plateau ($I_{ss}$), which can then be expressed as a percentage of peak response ($100[I_{ss}/I_p]$). Such a measure may be described as desensitization.

Figure 4.7 shows the degree of desensitization of AMPA currents in control, cholesterol-enriched and depleted neurones.

The influence of membrane cholesterol on the degree of desensitization of acutely dissociated neurones at near maximal AMPA exposure (300μM) was found to be statistically significant (% desensitization: 78.3±1.1, 87.2±1.4 and 65.1±2.3% respectively, for control, cholesterol-enriched and cholesterol-depleted neurones. These values were significantly different from each other ($p<0.05$, Dunnett test, two-way ANOVA).

![Figure 4.7](image)

**Figure 4.7** The percentage desensitization of AMPA receptor responses in control (n=18), cholesterol-enriched (n=19) and depleted (n=16) neurones.
4.2.4 Effect of cholesterol on the rate of current decay from peak

Although there is a large degree of variance in the decay of AMPA responses amongst neurones within the same preparation (Franke et al., 1993; Heckman & Dudel, 1997; Tour et al., 2000), it was of interest to investigate whether membrane cholesterol manipulation of acutely dissociated hippocampal neurones could alter the rate of decay for the enriched and depleted batches of neurones to such an extent so as to make it statistically significant.

The decay of the evoked currents in response to 300μM AMPA were therefore fitted by a single exponential with a time constant (τD), in the manner described by Tour et al., (2000), and the Chebyshev fitting method using Axon Clampfit (Chapter 2), thus giving a first-order approximation to the upper limit of the real time constant of desensitization (Table 4.4). An example of the single exponential fit of an AMPA response is shown in figure 4.8.

The effect of cholesterol enrichment was to speed up the rate of current decay and give a significantly lower value of the rate constant τD (Table 4.4). Conversely, cholesterol depletion slowed the rate of decay and gave a significantly higher value of τD.
Figure 4.8 AMPA (300μM) evoked whole cell currents in acutely dissociated hippocampal neurones showing the degree of decay in response in (A) control neurone vs enriched and (B) control vs depleted neurone. (C) The decay of a control current shown on an expanded time scale, fitted by a single exponential with a time constant ($\tau_D$).
Table 4.4  The effect of membrane cholesterol on AMPA current decay, shown as the rate constant ($\tau_D$) of a single exponential fit.

<table>
<thead>
<tr>
<th>Current decay</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_D$ (ms)</td>
<td>47.3±0.91</td>
<td>23.4±1.78</td>
<td>90.6±2.94</td>
</tr>
<tr>
<td>Sample number (n)</td>
<td>11</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>$P$ coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

43  Effect of membrane cholesterol on the current-voltage relationship

AMPA whole-cell current-voltage (I/V) relationships were constructed from the peak and steady-state responses evoked by 30µM AMPA in control, depleted and enriched neurones (Figure 4.9). The cells were voltage clamped between -60 to +60mV.

The depletion and enrichment of membrane cholesterol did not affect the AMPA I/V pots which were almost linear, with a slight outward rectification at large positive membrane potentials. The mean reversal potentials for control, depleted and enriched neurones were: -4.67mV, -3.2mV and -1.89mV for peak AMPA currents and -1.7mV, -1.1mV and +0.68mV, respectively, for plateau currents.
Figure 4.9 The peak (■) and steady-state (▲) current-voltage (I/V) relation of AMPA receptor mediated responses in whole cell recordings of hippocampal neurones. A=Control (n=10), B=Enriched (n=9) and C=Depleted (n=10) neurones.
4.4 Restoration of membrane cholesterol following depletion

In order to determine whether the effects of incubation with methyl-β-cyclodextrin were solely due to cholesterol depletion, rather than the depletion of other membrane components such as phospholipids, a cholesterol depletion step immediately followed by cholesterol repletion was investigated.

The reduced AMPA EC$_{50}$ values following cholesterol depletion could be restored by incubation with 0.15mM cholesterol-methyl-β-cyclodextrin complex (Table 4.6 and 4.7). This was indicative that the cholesterol-depletion procedure could be reversed. Depleted neurones which had undergone a repletion for 10, 15 and 20 minutes (Figure 4.10), had a cholesterol content of 92%, 163% and 194%, respectively (Table 4.5).

In an analogous manner to the enriched neurones (Figure 4.2), the raw maximal currents exhibited by the cholesterol-repleted neurones were found to be reduced compared to the maximum responses in depleted neurones (2285±251.9, 1427±196.2, and 1395±165.3 pA, respectively, for 10, 15 and 20 minutes repletion).

The AMPA EC$_{50}$ values for the peak and plateau responses as well as the Hill slope of the normalized dose-response curves for these neurones were also found to be increasingly similar to the neurones which had undergone cholesterol enrichment (section 4.2).

The AMPA concentration causing 50% desensitization in cholesterol-repleted neurones was 2.04±1.08 μM, compared to 7.52±0.66 μM in depleted neurones ($p<0.001$; Student’s $t$-test).
Table 4.5  The incubation of neurone samples with 5mM methyl-β-cyclodextrin (MβC) (depletion) or depletion followed by enrichment with cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC.

<table>
<thead>
<tr>
<th>Cholesterol content</th>
<th>Control</th>
<th>Depleted (10 mins)</th>
<th>Repleted (20 mins)</th>
<th>Repleted (15 mins)</th>
<th>Repleted (20 mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>µmole.mg protein⁻¹</td>
<td>0.429±0.03</td>
<td>0.277±0.08</td>
<td>0.396±0.05</td>
<td>0.686±0.09</td>
<td>0.805±0.11</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>64±1.7%</td>
<td>92±3.1%</td>
<td>163±2.6%</td>
<td>194±1.9%</td>
</tr>
</tbody>
</table>

Figure 4.10  Whole cell peak current responses to AMPA recorded from control, cholesterol-depleted and cholesterol-repleted hippocampal neurones clamped at -40mV.
Table 4.6  The effect of membrane cholesterol depletion followed by enrichment on peak AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Depleted (10 mins)</th>
<th>Repleted (15 mins)</th>
<th>Repleted (20 mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of neurones</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>EC50 (μM)</td>
<td>23.82±0.53</td>
<td>10.10±0.70</td>
<td>38.50±0.68</td>
<td>47.73±0.62</td>
</tr>
<tr>
<td>P coefficient (vs depleted)</td>
<td>&lt;0.01</td>
<td>-</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.34±0.07</td>
<td>1.02±0.09</td>
<td>1.15±0.14</td>
<td>1.42±0.10</td>
</tr>
</tbody>
</table>

Table 4.7  The effect of membrane cholesterol depletion followed by enrichment on plateau AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Depleted</th>
<th>Repleted (20 mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>2.36±0.19 (n=8)</td>
<td>3.98±0.27 (n=8)</td>
<td>1.98±0.36 (n=5)</td>
</tr>
<tr>
<td>P coefficient (vs depleted)</td>
<td>&lt;0.01</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.97±0.07</td>
<td>1.08±0.10</td>
<td>0.76±0.10</td>
</tr>
</tbody>
</table>
4.5 Effect of acute application of cholesterol on AMPA currents

Acute application of 1μM cholesterol, in the same U-tube solution as the applied AMPA, had no effect on the AMPA currents. The peak EC$_{50}$ values for the AMPA log.concentration-response relationship remained unchanged at 24.9±0.88 μM in the presence of cholesterol compared with 25.2±0.67μM in the control neurones. The plateau EC$_{50}$ was also not affected by cholesterol (2.53±0.24 and 2.6±0.31 μM for control and cholesterol application, respectively).

![Figure 4.11](image)

Figure 4.11 The effect of acute application of cholesterol on the AMPA concentration-response curves. Acute co-application of 1μM cholesterol with AMPA did not affect AMPA currents in acutely dissociated hippocampal neurones.
4.6 Discussion

The depletion of neuronal membrane cholesterol to 62% of control levels was found to result in a leftward shift of the log.concentration-peak response without change in the maximum agonist response, increasing the AMPA potency by 2.46 fold.

In contrast, increase in membrane cholesterol levels to 206% control, reduced the maximum of the AMPA log.concentration-peak response curve, while increasing the EC50 values, i.e. decreasing the potency of the agonist by a factor of 2.39.

Decay from the peak response to a plateau was found to be concentration related and reached a maximum at 100 AMPA μM and above. For investigation of the degree of decay from peak to plateau of AMPA receptor currents at different levels of membrane cholesterol content, receptor responses to 300μM AMPA were further analyzed. At close to maximum agonist response, receptor saturation minimizes the contribution of the unbound state so that the rise of current mainly reflects the bound-open equilibration, while the decay represents the equilibration into desensitized states. At lower concentrations of agonist, channel activity is highly unsynchronized since some channels are opening or desensitizing, while others are still waiting to bind agonist.

Although the speed of solution change in the present study did not allow for a detailed evaluation of the fast kinetics, cholesterol-enriched neurones were on the whole found to desensitize faster and to a greater extent, compared to control and depleted neurones. The latter showed a much slower onset of desensitization, as well as a diminished degree of fade to plateau, even at the highest concentrations of the agonist. There was a strong correlation between the size of the plateau response from whole-cell recordings and the
desensitization rate of the neurones, suggesting that the speed and extent of the decay were inextricably linked.

The Hill coefficient was another parameter that was altered by the cholesterol-status of the neurones. Cholesterol-enriched neurones were found to have a steeper Hill slope compared to control and cholesterol-depleted neurones. The Hill equation has the property that when drawn as a Hill plot \( \log\left(\frac{(y-y_{\text{min}})}{(y_{\text{max}}-y)}\right) \) against \( \log([A]) \), it produces a straight line with a slope that is the Hill coefficient, denoted \( n_H \). This slope will be steep if the agonist binding shows cooperativity (higher affinity for the second binding than the first), conversely will be reduced if the second binding shows lower affinity (Colquhoun, 1998). An increase in the Hill coefficient, observed in cholesterol-enriched neurones, may therefore be indicative of a greater affinity for the second binding (double-ligand activation). Conversely, when the second binding is weakened, then the Hill slope is reduced (cholesterol-depletion). This is potentially informative, but the Hill equation is an empirical description, making it difficult to deduce definitive conclusions.

The agonist-evoked peak current is a convenient parameter to quantify receptor responses. The peak represents the instant when recruitment of new ionophores to conducting states is exactly balanced by the passage of others into the desensitised phase (Patneau et al., 1993). Changes in either of these factors would be expected to alter the amplitude of the peak receptor response (Ip). When comparing the AMPA receptor responses of cholesterol-depleted and enriched neurones, the results of this study indicate that AMPA potency is inversely related to the rate of desensitisation. Given that
only a fraction of $I_p$ is reduced by desensitisation (Partin et al., 1996; Tour et al., 2000), the question arises of whether the changes in desensitisation due to cholesterol enrichment and depletion fully or partially account for the changes in peak $EC_{50}$ values.

There is evidence that binding of agonist to a single AMPA subunit is sufficient for channel opening (Rosenmund et al., 1998; Partin, 2001). But although domains within individual AMPA subunits can influence desensitisation, the extent to which subunit-subunit interaction or dimer configurations play a role is less clear.

It is also important to note that desensitisation can occur both from fully liganded and the partly liganded states. The rate of desensitisation for the single-liganded state differs from the fully liganded state in that the rate constant of desensitisation from the former state has been reported to be much larger than the latter (Heckman and Dudel, 1997). Data derived from Axoclamp recordings (Table 4.4) shows a slower rate of receptor desensitisation in the depleted neurones, as opposed to the enriched cells, suggesting that lower cholesterol levels may encourage single-liganded activation. This is in direct agreement with the lowering of the Hill coefficient to unity (Table 4.2), which was observed with cholesterol-depleted neurones, and vice-versa for states of cholesterol enrichment. However, this binding behaviour can only be positively confirmed by performing ligand-binding studies.

AMPA channel recovery from desensitised states is typically slow and continues even after removal of glutamate and channel closure (Hestrin, 1992; Raman and Trussell, 1995), suggesting that recovery from desensitisation is a rate limiting step in the return
to the unbound state (Jones and Westbrook, 1996). The recovery may be further hindered in conditions of low membrane fluidity such as in enriched neurones, reducing the number of AMPA receptors available for reactivation.

The term 'membrane fluidity' is a semiquantitative term encompassing all molecular motions throughout the membrane bilayer such as lateral and rotational motions, trans-gauche isomerizations, and anisotropic motions (Fong and McNamee, 1986). Changes in membrane fluidity can influence receptor binding behaviour and gating of the associated channel (Klein et al., 1995; Addona et al., 1998). In a control neurone, the binding of agonist to the receptor and the subsequent movement of the binding pocket produces tension on the connecting portion between the extracellular binding pocket and the receptor's transmembrane domains. This leads to conformation changes within the membrane, forcing the channel to open (Rosenmund & Mansour, 2002).

In a depleted neurone, an increased membrane fluidity may facilitate conformational changes of the receptor by reducing the energy barrier, whereas enrichment can result in a more rigid membrane structure. Physical strain could also be directly transferred to the AMPA receptor channel and conceivably influence receptor response by altering the affinity of the ligand binding site. This mechanism could alter AMPA potency independent of any additional influence of desensitisation.

Figure 4.12 exhibits a model depicted to show the ability of cholesterol-depleted neurones to stabilize the open channel state following the activation of AMPA receptors. The model assumes that activation of the channel and the subsequent conformational
change, results in a net asymmetric expansion in the two leaflets of the membrane’s bilayer, causing an increase in the mechanical strain of the bilayer. Relief of this strain can be achieved by rapid flipping of cholesterol molecules from the upper leaflet to the lower leaflet of the bilayer (Sunshine and McNamee, 1992). This facilitates the conformation change to proceed and stabilizes the activated channel, implying that the conformational-state interconversions are dependent on the nature of the lipid environment.

Figure 4.12  Schematic model demonstrating a possible method for the relief of the conformation-induced bending strain in the lipid bilayer experienced upon binding of the agonist in cholesterol-depleted neurones. Figure adapted and modified (Addona et al., 1998) showing: resting membrane (a), and dynamics following AMPA binding (b and c).

Additionally, the affinity-state transitions involve conformational changes that lead to receptor desensitisation. Such a dynamic process is expected to be influenced by the dynamic structure of membrane since the ionic channel is located within the membrane.

Membrane cholesterol depletion or enrichment altered neither the shape nor the reversal potential of whole cell AMPA current-voltage relationship, indicating that the effect of
membrane cholesterol on AMPA EC\textsubscript{50} was not voltage dependent and that the channels possessed a similar permeability to the relevant cations.

The shape of the current-voltage plots and the corresponding rectification and divalent permeability of AMPA mediated GluR channels, measured with AMPA-activated whole cell currents, depend on the cell type examined. It must be noted that both inward and outward rectifications were observed in all three membrane cholesterol states. The roughly linear current-voltage relations of the whole cell currents are thought to be a reflection of the average behaviour of a heterogeneous population of AMPA receptors, rather than reflecting the rectification behaviour of a single type of AMPA subset.

Amongst acutely dissociated hippocampal neurones, heteromeric channels may coexist in the same cell with homomeric channels with high divalent permeability, suggesting that the AMPA-activated divalent permeability of single cells may represent the superposition of currents mediated by a mosaic of both divalent impermeable heteromeric and divalent permeable homomeric AMPA receptor channels (Hume at al. 1991; Lerma et al., 1994; Banke and Lambert, 1999; Dingledine et al., 1999).

With regards to the restoration of membrane cholesterol following depletion, the recovery of cholesterol levels (Table 4.5 and 4.6) excluded the possibility of membrane phospholipid perturbation contributing to the effects of cholesterol depletion. This study was essential as the distribution of phosphatidylserine in membranes has been found to modulate the properties of AMPA receptors. Altered levels of this phospholipid can influence the binding of [\textsuperscript{3}H]-AMPA which includes enhanced ligand binding at high membrane concentrations of phosphatidylserine. It must be noted that
Phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and cholesterol, however, were not found to modify $[^3]$H-AMPA binding at concentrations up to 1.5mg/mg protein (Baudry et al., 1991).

The question which remained unanswered at this stage was that although the samples of the neurones which had undergone repletion for 10 minutes were found to have membrane cholesterol levels of 92% (Table 4.5), their corresponding AMPA EC$_{50}$ values were higher than those of the control by 1.6 fold. Also, although the maximum raw currents exhibited by the cholesterol-repleted neurones (20 minutes), were lower than those recorded for the neurones subjected to direct enrichment, the EC$_{50}$ values remained comparable. The normalized dose-response curves showed an incubation-dependent rise in the Hill slopes, but these values did not reach the level of direct enrichment.

The possibility therefore remained that, beside its role as a bilayer constituent, cholesterol could play a complex role as an allosteric effector of the AMPA receptor by acting on the protein regulatory sites at the lipid-protein interface of the receptor.

The next stage of this investigation, therefore, involved a search for a cholesterol analogue, which could be incorporated into the plasma membrane, and the study of the effects of such a substitution on the AMPA receptor response.
Chapter 5

Effect of Epicholesterol on the Sensitivity of AMPA Receptors to AMPA
Chapter 5

5.1 Introduction

In the studies of the influence of membrane cholesterol on the properties of the oxytocin and cholecystokinin receptors, Gimpl et al., (1997) have extensively explored the consequences of replacing some of the membrane cholesterol with other related sterols. They have defined rather precise structural requirements for sterols to mimic cholesterol’s influence on the oxytocin receptor, with less stringent requirements with regard to the cholecystokinin receptor.

In studies on some membrane enzymes that are dependent on membrane cholesterol for their activity, substitution of the cholesterol by some closely related sterols did not restore the enzyme activity. For example, epicholesterol, androstenol, dihydrocholesterol, lanosterol and ergosterol could not restore the activities of the Na^+, K^+-ATPase and Na^+-Ca^{2+} exchanger (Vermuri and Phillipson, 1989); cholest-4-en-3-one, dehydroisandrosterone, campesterol, desmosterol or 5α-androstan-3β-ol could not restore the activity of GABA transporter (Shouffani and Kanner, 1990).

Using the current methodology, the choice of a cholesterol substitute needed to account for the fact that during the incubation of the membranes with sterol-methyl-β-cyclodextrine complexes, the sterol content of the inclusion complex would progressively diminish, according to the equilibrium of sterols between donor and acceptor. So, the initial donor sterol-methyl-β-cyclodextrine could also function as a cholesterol acceptor, leading to some cholesterol-depletion of the membranes, if an inefficient steroid exchanges for cholesterol.
While studying the properties of several steroids, Gimpl et al., (1997) have shown that epicholesterol, the 3α-hydroxy isomer of cholesterol, can be satisfactorily incorporated into the neuronal membrane and can adequately substitute for cholesterol in maintaining membrane fluidity as measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence anisotropy.

To further investigate the influence of cholesterol on the AMPA receptor response, it was therefore decided to repeat the cholesterol manipulation exercise while using epicholesterol (5-cholestene-3α-ol), as a substitute.
5.2 Effect of incubation with epicholesterol on AMPA responses

Epicholesterol was added to the plasma membrane of control neurones for sterol enrichment or used to replace cholesterol in the cholesterol-depleted neurones by incubation of the neurones with epicholesterol-methyl-β-cyclodextrin complex.

The degree of enrichment for epicholesterol-enriched neurones could not be subjected to the same type of analysis as cholesterol due to the lack of a quantitative detection method for epicholesterol. Reliance has therefore been placed on the work of Gimpl et al. (1997), which shows that epicholesterol incorporates into plasma membranes just as readily as cholesterol from the methyl-β-cyclodextrin vehicle.

The cholesterol contents of the neurones used in this part of the study are shown in Table 5.1.

Table 5.1 The cholesterol content of neurone samples following incubation at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or epicholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM epicholesterol + 1.5mM MβC (enrichment).

<table>
<thead>
<tr>
<th>Cholesterol content</th>
<th>Control</th>
<th>Depleted</th>
<th>Epicholesterol-enriched</th>
<th>Depleted + Epi-enriched (10 minutes)</th>
<th>Depleted + Epi-enriched (20 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample number</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>µmole.mg protein⁻¹</td>
<td>0.341±0.014</td>
<td>0.219±0.043</td>
<td>0.337±0.011</td>
<td>0.216±0.057</td>
<td>0.225±0.028</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>65±2.6%</td>
<td>98±0.72%</td>
<td>63±3.8%</td>
<td>66±1.9%</td>
</tr>
</tbody>
</table>
5.2.1 Effect of epicholesterol enrichment on peak AMPA currents

Incubation of neurones with 0.15 mM epicholesterol-methyl-β-cyclodextrin was found to result in a reduced maximum response to AMPA (2533±172.6 pA and 1478±149.3 pA for control and epicholesterol-enriched neurones, respectively (Figure 5.1).

The normalised dose-response curve (Figure 5.2) also showed an increase in the peak AMPA EC$_{50}$ values (Table 5.2).

The incubation of cholesterol-depleted neurones with 0.15 mM epicholesterol-methyl-β-cyclodextrin complex for 10 and 20 minutes (Figure 5.3) also resulted in increases of AMPA EC$_{50}$ values in the same manner to cholesterol enrichment following depletion, discussed in Chapter 4. The raw maximum AMPA currents were 2836±197.5 pA, 1669±252.2 pA and 1141±209.5 pA, for depleted neurones and epicholesterol-repleted neurones following 10 and 20 minute incubations, respectively.

![Figure 5.1 AMPA dose-response relationships showing whole cell peak current responses to AMPA, recorded from control and epicholesterol-enriched acutely dissociated hippocampal neurones, clamped at -40mV.](image-url)
Table 5.2  The effect of membrane epicholesterol enrichment on peak AMPA dose-response relationships of acutely dissociated neurones. Numbers of experiments are shown in parentheses.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Epicholesterol enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of neurones</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Peak current EC$_{50}$ (µM)</td>
<td>24.63±0.72</td>
<td>48.47±1.92</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.38±0.05</td>
<td>1.56±0.04</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 5.3  The effect of membrane cholesterol depletion and depletion followed by epicholesterol enrichment (10 and 20 minutes) on peak AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + Epi-enriched (10 minutes)</th>
<th>Depleted + Epi-enriched (20 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of neurones</td>
<td>7</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Plateau current EC$_{50}$ (µM)</td>
<td>9.84±0.56</td>
<td>43.27±1.45</td>
<td>59.38±1.33</td>
</tr>
<tr>
<td>P coefficient (vs depleted)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.05±0.08</td>
<td>1.44±0.10</td>
<td>1.72±0.10</td>
</tr>
<tr>
<td>P coefficient (vs depleted)</td>
<td>-</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 5.2 Whole cell peak current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mv. (■) Control neurones (n=9) and (▲) epicholesterol-enriched neurones (n=12).

Figure 5.3 Whole cell peak current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mv. (■) Control neurones (n=7), (▲) cholesterol-depleted neurones (n=7), and (▲) depletion followed by epicholesterol enrichment (20 minutes; n=9).
5.2.2 Effect of epicholesterol on plateau AMPA currents

The enrichment of acutely dissociated neurones resulted in a statistically significant reduction in the plateau response $EC_{50}$ values compared to control (two-way ANOVA) (Table 5.3). Similarly, epicholesterol enrichment of cholesterol-depleted neurones significantly reduced the plateau $EC_{50}$ values (Figure 5.4) compared to those of depleted neurones (Table 5.3).

![Graph A](image1.png) ![Graph B](image2.png)

**Figure 5.4** Whole cell plateau current responses to AMPA recorded from dissociated hippocampal neurones clamped at -40mV. A) (▲) epicholesterol enriched neurones (n=12) vs. control (dotted line) (n=9). B) (▲) cholesterol depletion followed by epicholesterol enrichment (20 minutes, n=9) vs. depleted neurones (dotted line) (n=7).
### Table 5.4  The effect of membrane epicholesterol enrichment on plateau AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Epichol-enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of neurones</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Plateau current EC₅₀</td>
<td>2.43±0.26μM</td>
<td>1.89±0.23μM</td>
</tr>
<tr>
<td>P coefficient (vs control)</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.89±0.10</td>
<td>0.77±0.18</td>
</tr>
</tbody>
</table>

### Table 5.5  The effect of membrane cholesterol depletion and depletion followed by epicholesterol enrichment (10 and 20 minutes) on plateau AMPA dose-response relationships of acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + Epi-enriched (10 minutes)</th>
<th>Depleted + Epi-enriched (20 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of neurones</td>
<td>7</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Plateau current EC₅₀</td>
<td>4.48±0.31μM</td>
<td>3.06±0.98</td>
<td>2.72±0.40μM</td>
</tr>
<tr>
<td>P coefficient (vs depleted)</td>
<td>-</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.96±0.13</td>
<td>0.83±0.22</td>
<td>0.75±0.22</td>
</tr>
</tbody>
</table>
5.2.3 **Effect of epicholesterol on the decay from peak to plateau AMPA currents**

The concentration of AMPA causing 50% desensitization in epicholesterol-enriched neurones (2.84±0.97 µM) was comparable to the desensitization of the cholesterol-enriched neurones (Chapter 4).

The change in AMPA desensitization in epicholesterol-repleted neurones, following 10 and 20 minute (Figure 5.5) incubations, were also statistically significant, compared to cholesterol-depleted neurones (50% desensitization: 3.14±0.98, 1.97±1.14 and 7.33±0.62 µM, respectively; p<0.01, two-way-ANOVA).

*Figure 5.5* The percentage desensitization of AMPA receptor responses in depleted (*n=7*) and epicholesterol-repleted (20 minute incubations, *n=12*) neurones.
5.3 Discussion

The presence of excess sterol in the membrane, in the form of epicholesterol, resulted in a reduction of maximum current responses to AMPA in a similar manner to cholesterol enrichment.

The normalised log-concentration response curves also displayed a rightward shift in epicholesterol-enriched states, indicating that the AMPA receptors required a 1.97 fold higher agonist concentration for activation.

The resultant increase in the value of the Hill slope was however less pronounced compared to cholesterol-enriched neurones discussed in the previous chapter.

Epicholesterol enrichment enhanced the desensitisation of AMPA receptors, exhibiting a maximum degree of desensitization of 83.6 ± 1.7%.

Measurement of the total cholesterol content, following the epicholesterol-enrichment process, showed that there was no further reduction in the cholesterol level of these neurones (Table 5.1).

Since the depletion of neurones was shown to remove about 40% of the cholesterol contained in the neuronal membranes, any direct alteration in AMPA receptor behaviour in the presence of epicholesterol could be better differentiated from those of cholesterol, following the depletion process.

The overall influence of sterol repletion in cholesterol-depleted states, although analogous in terms of lower absolute currents displayed and reduced AMPA potencies, was found to be more pronounced with epicholesterol-repletion, compared to cholesterol-repletion. The greater macroscopic desensitization in the presence of
epicholesterol was once again believed to contribute to the lowering of the evoked currents. The change in the Hill coefficient only became significant following the longer repletion period of 20 minutes (p<0.05, compared to control).

From a fluidity point of view, it can be argued that an expectation of identical behaviour to cholesterol may not be reasonable, as it is possible that the sterol load, using different substitutions, could lead to receptor independent changes in the membrane, such as changes in packing order (Rousselet et al., 1981).

There is little doubt that the dynamic properties of the neuronal membrane can have a great impact on receptor properties. However, given that the agonist binding site/s of AMPA receptors are located on the extracellular domain (Figure 1.4), well above the surface of membranes (Armstrong and Gouaux, 2000), the subunit binding sites were not believed to be directly influenced by the change of molecular interactions at the lipid-protein interface. As discussed in Chapter 1, the S1/S2 binding domain is also of primary importance in the control of AMPA desensitization and its modulation by drugs (Partin, 2001; Sun et al., 2002).

The data collected so far, imposed an inevitable limitation in drawing comparisons amongst the batches of neurones, in that the effect of altered sterol levels could not be studied within the same neurone.

The similarities between the influences on the AMPA receptor function, however, suggested that if specific sterol binding sites on the AMPA receptor are present, then a
strict structural requirement did not apply in terms of the stereospecificity of the hydroxyl group at the C3 position.

The steroids present within the CNS, which are ultimately derived from cholesterol, share some structural and functional similarities to the parent substrate.

In an attempt to further study the influences of sterols on the AMPA receptor function, it was decided to direct the investigations towards other possible steroid modulators.
Chapter 6

Effect of Neuroactive Steroids on the Sensitivity of AMPA Receptors to AMPA
6.1 Introduction

Since the discovery that some hormonal steroids can be synthesized de novo from cholesterol in the brain and the peripheral nerves, much research has been directed towards the mechanism of action and the specific targets of such neurosteroids.

The best-studied neuroactive steroids are a series of 3α-hydroxy ring A-reduced pregnane steroids. These 3α-hydroxysteroids do not generally interact with classical intracellular steroid receptors but have been shown to bind stereoselectively and with high affinity to receptors such as GABA$_A$ (Lambert et al., 1995; Sooksawate & Simmonds, 2001). The modulation of neurotransmitter receptors by such steroids is however not restricted to GABA$_A$ receptors (refer to introduction in Chapter 1). It has therefore been proposed that neuroactive steroids may play a physiological, or pathophysiological role to locally modulate neuronal excitability by rapid “fine tuning” of the actions of membrane ligand-gated receptors. Attempts are also being made to exploit such findings for therapeutic advantage (Gasior et al., 1999; Goodchild et al., 2001; Lambert et al., 2001).

The structure-activity requirements for the modulation of ligand-gated ion channels by neuroactive steroids differ considerably between members of these neurotransmitter receptor families, indicating that the non-genomic behaviour is not a generic membrane effect (Rupprecht et al., 2001).

There is currently very little published data regarding the possible rapid modulation of the AMPA subset of the glutamate receptors by neuroactive steroids. Therefore efforts were made to study a range of steroids for their ability to influence the AMPA receptor responses in acutely dissociated hippocampal neurones.
6.2 Selection of AMPA receptor modulators

A “screening” process was set up to differentiate the steroidal compounds which displayed a rapid modulation of AMPA receptors at concentrations of 0.1, 1 and 10μM, following a three minute incubation period.

It was found that: cortisol (11β, 17α, 21-Trihydroxypregn-4-ene-3,20-dione; 17-hydroxycorticosterone; also known as hydrocortisone), pregnanolone (5β-pregn-3α-ol-20-one), epipregnanolone (5β-pregnan-3β-ol-20-one), testosterone (4-androsten-17β-ol-30one), 5β-THDOC (5β-pregnane-3α,21-diol-20one; 21-hydroxypregnanolone; tetrahydro DOC: tetrahydrodeoxycorticosterone;) and DHEAS (5-Androsten-3β-ol-17-one-3-sulphate; dehydroepiandrosterone sulphate) did not have a rapid modulatory influence on AMPA receptor responses.

However, PS (3β-hydroxy-5-pregnen-20-one-3-sulphate; pregnenolone sulphate), CORT (4-pregnene-11β,21-diol-3,20-dione; corticosterone), DOC (4-pregnene-21-ol-3,20-dione; deoxycorticosterone), 5α-THDOC (5α-pregnane-3α,21-diol-20one; 21-Hydroxyallopregnanolone; Allotetrahydrodeoxycorticosterone), allopregnanolone (5α-pregnan-3α-ol-20-one) and epiallopregnanolone (5α-pregnan-3β-ol-20-one; 3β-hydroxy-5α-pregnan-20-one; 3β-OH DHP) displayed various degrees of modulation of AMPA receptor currents at concentrations of 0.1-10μM.

Progesterone (4-pregnene-3,20-dione) did not modulate AMPA EC50 values at these concentrations and only exerted a statistically significant inhibitory influence at concentrations of 20μM or above.
Figure 6.1  The structures of the main neuroactive steroids outlined in this chapter.
6.3 Effect of cortisol on AMPA receptor responses

Application of 1 and 10 μM cortisol, in the same U-tube solution as the AMPA, had no effect on the AMPA evoked currents.

The peak EC\textsubscript{50} values for the AMPA log.concentration-response relationship remained statistically unchanged at 26.64±0.93 μM in the presence of 10 μM cortisol compared with 25.12±0.35 μM in the control neurones. The plateau EC\textsubscript{50} was also not affected by cortisol application (2.48±0.19 and 2.55±0.21 μM for control and in presence of 10 μM cortisol, respectively).

![Graph](image.png)

**Figure 6.2** The effect of application of 10μM cortisol on the AMPA concentration-response curve in acutely dissociated hippocampal neurones (n=8). (■) control neurones and (◇) control + 10 μM cortisol.
6.4 Modulation of AMPA receptor responses by pregnenolone sulphate

The naturally occurring sulphate ester of the 3β-hydroxysteroid pregnenolone has a wide range of effects on membrane channels at concentrations in the high nanomolar to micromolar range, which frequently exceeds those of the free steroid (Baulieu, 1998).

The effect of 0.1, 1 and 10μM pregnenolone sulphate on 30 μM AMPA responses on a control neurone is shown in figure 6.3. Pregnenolone sulphate caused a reduction in peak and plateau amplitudes of AMPA currents in a concentration-dependent manner.

Exposure to increasing concentrations of pregnenolone sulphate resulted in a rightward shift of the AMPA dose-response curves for peak currents (Figure 6.4), with a small increase in Hill slope and a reduction of the maximum response (Table 6.1).

Similarly, the calculated EC50 values for plateau currents were found to increase in the presence of pregnenolone sulphate resulting in higher AMPA EC50 ratios with increasing concentrations of pregnenolone sulphate (Table 6.2).

In three of the recorded neurones, however, the plateau currents were potentiated so as to increase the Iss/Ip ratio to unity. As previously reported, the modulation of the plateau currents varied considerably amongst different dissociated neurones.

Figure 6.5 demonstrates the degree of desensitization of AMPA currents in the combined presence of agonist and 10μM pregnenolone sulphate, expressed as the percentage ratio of steady-state current (Iss) to peak current (Ip) (refer to section 4.3), compared to agonist alone. The corresponding desensitization curve, showed a decrease in the AMPA concentration required to cause 50% desensitization in neurones exposed
to the neurosteroid (1.74±0.43 µM), compared to control values (3.97±0.28 µM). This was found to be statistically significant (p<0.001, Student’s t-test).

As pregnenolone sulphate has been shown to be a potent negative modulator of GABA<sub>A</sub> receptors (Sooksawate & Simmonds, 2001), it was decided to repeat these experiments in the presence of picrotoxin, a non-competitive GABA<sub>A</sub> antagonist, in order to investigate the possibility of a GABAergic influence on the neuronal responses. This was in view of the reported release of GABA in the presence of glutamate agonists (Ben-Ari et al., 1997), and also data from investigations by Richardson & Wakerly (1998), which pointed at a reversal of the suppressive action of pregnenolone sulphate on AMPA-stimulated firing in the presence of 50µM picrotoxin, in the study of the activity of supraoptic oxytocin and vasopressin neurones.

The AMPA dose-response curve, in the presence of 10µM pregnenolone sulphate and 10µM picrotoxin, gave a peak AMPA EC<sub>50</sub> ratio of 1.83±0.67 (n=7), compared to 1.86±0.13 in the absence of picrotoxin. The difference was not found to be statistically significant. Picrotoxin alone did not have any influence on the AMPA dose-response curve (EC<sub>50</sub>=25.37±0.59 µM; p>0.05, Student’s t-test).
Figure 6.3  AMPA (30 \mu M) evoked whole cell currents in a control acutely dissociated neurone (A), in the presence of varying concentrations of PS (B-D), and following a washout period of 3 minutes (E).
Table 6.1 The effect of PS on peak AMPA dose-response relationships of acutely dissociated neurones (n=8, 8 and 21 for 0.1, 1 and 10 μM PS respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>PS (0.1μM)</th>
<th>PS (1 μM)</th>
<th>PS (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (μM)</td>
<td>24.09±0.85</td>
<td>28.65±1.94</td>
<td>32.13±2.07</td>
<td>44.83±2.65</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.19±0.06</td>
<td>1.33±0.10</td>
<td>1.86±0.13</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.37±0.03</td>
<td>1.44±0.08</td>
<td>1.52±0.07</td>
<td>1.93±0.11</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±1.06</td>
<td>91.18±1.10</td>
<td>83.34±1.80</td>
<td>65.72±1.73</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 6.2 The effect of PS on plateau AMPA dose-response relationships of acutely dissociated neurones (n=8, 8 and 21 for 0.1, 1 and 10 μM respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>PS (0.1μM)</th>
<th>PS (1 μM)</th>
<th>PS (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (μM)</td>
<td>2.72±0.19</td>
<td>3.10±1.08</td>
<td>3.47±0.92</td>
<td>4.46±0.85</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.14±0.05</td>
<td>1.28±0.10</td>
<td>1.64±0.12</td>
</tr>
</tbody>
</table>
Figure 6.4  AMPA dose-response relationships showing whole cell peak current responses to AMPA alone (■) and in presence of 0.1 μM PS (▼) (n=8), 1 μM PS (▼) (n=8) and 10 μM PS (▼) (n=21), recorded from dissociated hippocampal neurones clamped at -40mV.

Figure 6.5  The degree of fade to plateau of control AMPA receptors expressed as the percentage desensitisation in control neurones (dotted line), and control neurones + 10μM PS (▼).
Because pregnenolone sulphate is charged, voltage-dependence of inhibition would be expected if access to its binding site requires entry into the channel. Figure 6.6 shows the peak current-voltage (I/V) relationship of AMPA receptor mediated responses of control neurones prior to and after exposure to 10 μM pregnenolone sulphate (n=9). The corresponding I/V plot remained linear, with no statistically significant change in the mean reversal potential of peak AMPA currents (-4.57 and -4.62 mV in the presence of the steroid compared to control, respectively).

Separate experiments were also carried out by incorporation of 0.3 μM Pregnenolone sulphate (n=11) within the electrode buffer, so as to study an intracellular application of the steroid. A low intracellular concentration was chosen in order to keep the use of the solvent, DMSO, to a minimum. The AMPA receptor responses did not vary significantly from control neurones, patched with electrodes not containing PS, and remained stable throughout the recording period. The resulting dose-response relationship for neurones patched with 0.3 μM PS yielded an EC50 of 26.04 ± 1.05 μM (n=11), which was not statistically different from the controls (25.23 ± 0.76 μM) (p>0.05, Student’s t-test).

Figure 6.6 The peak current-voltage (I/V) relation of AMPA receptor mediated responses in whole cell recordings of control hippocampal neurones (■) and in the presence of 10 μM pregnenolone sulphate (■).
6.5 Modulation of AMPA receptor responses by deoxycorticosterone and corticosterone

The attenuation of 30μM AMPA currents by deoxycorticosterone in a control acutely dissociated neurone is shown in figure 6.7. The presence of 0.1 and 1μM deoxycorticosterone resulted in a reduction of peak and plateau AMPA currents. At 10μM concentration, however, the neuroactive steroid caused an extensive reduction of peak amplitude, while reducing the plateau current to a much smaller extent.

Figure 6.8 shows the normalized AMPA log.concentration-response relationship of control neurones at varying concentrations of deoxycorticosterone. The presence of the steroid resulted in a shift of the dose-response curve for peak responses to the right with an increase in Hill slope and a reduction of the maximum response, which was enhanced at higher concentrations of 1 and 10μM deoxycorticosterone (Table 6.3). The dextral shift yielded an increase in peak AMPA EC\textsubscript{50} ratios with increasing concentrations of the neurosteroid (Table 6.3).

The plateau responses appeared similarly affected with respect to the EC\textsubscript{50} values which were found to be increased, yielding increased AMPA EC\textsubscript{30} ratios in the presence of deoxycorticosterone (Table 6.4).

The percentage desensitization of the AMPA currents in the presence of 10 μM deoxycorticosterone is shown in Figure 6.9. The effect of 1 and 10 μM deoxycorticosterone on the AMPA concentration at which the 50% desensitization was obtained (2.39±0.71 and 2.07±0.53 μM, respectively) was found to be statistically significant, compared to control neurones (3.86±0.42 μM; p<0.001, Student’s t-test).
Figure 6.7 AMPA (30 μM) evoked whole cell currents in a control acutely dissociated neurone (A), in the presence of varying concentrations of deoxyorticosterone (DOC) (B-D), and following a washout period of 3 (E) and 5 minutes (F).
Chapter 6

Table 6.3  The effect of DOC on peak AMPA dose-response relationships of acutely dissociated neurones (*n*=7, 11 and 16 for 0.1, 1 and 10µM respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>DOC (0.1µM)</th>
<th>DOC (1µM)</th>
<th>DOC (10 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>25.22±0.86</td>
<td>32.03±1.73</td>
<td>41.74±2.15</td>
<td>52.69±3.08</td>
</tr>
<tr>
<td>( P ) coefficient (vs paired control)</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.27±0.08</td>
<td>1.65±0.11</td>
<td>2.09±0.17</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.40±0.03</td>
<td>1.51±0.07</td>
<td>1.62±0.12</td>
<td>1.66±0.09</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±1.12</td>
<td>82.49±3.09</td>
<td>53.56±2.54</td>
<td>44.06±2.07</td>
</tr>
<tr>
<td>( P ) coefficient (vs paired control)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.4  The effect of DOC on plateau AMPA dose-response relationships of acutely dissociated neurones (*n*=7, 11 and 16 for 0.1, 1 and 10µM respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>DOC (0.1µM)</th>
<th>DOC (1µM)</th>
<th>DOC (10 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>2.64±0.28</td>
<td>2.93±0.76</td>
<td>3.83±0.85</td>
<td>4.02±2.13</td>
</tr>
<tr>
<td>( P ) coefficient (vs paired control)</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.11±0.04</td>
<td>1.45±0.06</td>
<td>1.52±0.10</td>
</tr>
</tbody>
</table>
Figure 6.8 AMPA dose-response relationships showing whole cell peak current responses to AMPA alone (■) and in presence of 0.1\,\mu M DOC (▼), 1\,\mu M DOC (▼) and 10\,\mu M DOC (▼) recorded from dissociated hippocampal neurones clamped at -40\,mV.

Figure 6.9 The degree of fade to plateau of control AMPA receptors expressed as the percentage desensitisation in control neurones (dotted line), and control neurones + 10\,\mu M DOC (▼).
The glucocorticoid corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) has a similar structure to deoxycorticosterone, with the addition of a hydroxyl group at C-11.

The AMPA log.concentration-response curve in the presence of 10 μM corticosterone (n=4), showed a reduction in the maximum response (69.12±3.55%), as well as an increase in peak and plateau EC$_{50}$ values (38.56±2.92 μM and 3.16±1.64 μM, respectively).

The % desensitisation of AMPA receptor responses in the presence of 10 μM corticosterone was statistically different from control values (AMPA concentrations of 2.85±1.15 μM and 3.62±0.97 μM, respectively, yielding 50% desensitization).

Corticosterone was found to have an overall smaller inhibitory influence on AMPA receptor responses compared to deoxycorticosterone.
6.6 Modulation of AMPA receptor responses by progesterone

Exposure of neurones to 0.1 and 1 μM progesterone, in the same solution as AMPA, did not alter the agonist-evoked currents in hippocampal neurones. However, 10 and 20 μM applications, caused a reduction of the maximum response of the resultant log.concentration-response curves (91.62±1.43% and 82.16±2.11%, for 10 and 20 μM progesterone, compared to 98.94±1.27% in control). The exposure to 20 μM progesterone also resulted in a rightward shift of the curve (Figure 6.10), and hence an increased peak AMPA EC₅₀ (29.94±1.28 μM) in the presence of 20 μM progesterone, compared with 25.07±0.56 μM in the control neurones (n=8), p<0.05, Student’s t-test).

The plateau EC₅₀ was also increased by progesterone application (2.40±0.17 and 2.87±0.23 μM for control and in presence of 20 μM progesterone, respectively; p<0.05).

The influence of 20 μM progesterone on the percentage desensitization of AMPA receptor responses was found to be statistically significant. 50% desensitization was obtained with 3.08±0.89 μM AMPA in the presence of progesterone, compared to 3.91±0.61 μM AMPA in control neurones (p<0.01, Student’s t-test).
Figure 6.10 AMPA dose-response relationships showing whole cell peak current responses to AMPA alone (■) and in presence of 20μM progesterone (▼) (n=8), recorded from dissociated hippocampal neurones clamped at -40mV.
Chapter 6

6.7 Modulation of AMPA receptor responses by THDOC

An example of the currents induced by 30\(\mu\)M AMPA on a control neurone, prior to and in the presence of 0.1, 1\(\mu\)M and 10\(\mu\)M THDOC, displays an attenuation of the evoked responses by the neurosteroid which was overcome following a wash out period of approximately 5 minutes (Figure 6.11). At all three concentrations of THDOC, both the peak and plateau components of the AMPA currents were reduced.

The resulting dose-response curve for peak AMPA responses, shown in figure 6.10, was shifted to the right with an increase in Hill slope and no apparent change in the maximum responses (Table 6.5), indicating that the neurosteroid antagonism was overcome at high agonist concentrations. High concentrations of THDOC (10\(\mu\)M or more) were found to be required for a clear effect, but a statistically significant inhibition was nevertheless seen with concentrations as low as 0.1\(\mu\)M (Table 6.5).

The plateau responses were similarly affected, leading to an increase in plateau EC\(_{50}\) ratios with increasing concentrations of the neurosteroid (Table 6.6).

The percentage desensitization of the AMPA currents in the presence of 10 \(\mu\)M THDOC is shown in Figure 7.13. The influence of 10 \(\mu\)M THDOC on the AMPA concentration responses required for 50% desensitization, was not found to be statistically significant (3.94±0.47 and 3.72±0.58 \(\mu\)M, respectively; \(p>0.05\), Student's \(t\)-test).

In the same manner as pregnenolone sulphate, the mean reversal potential of peak AMPA currents in the presence of 10 \(\mu\)M THDOC (n=4) remained unchanged. Also, the intracellular application of 0.3 \(\mu\)M THDOC (n=7), did not result in a statistically
significant change in the resultant EC\(_{50}\) values, in the absence of extracellularly applied steroid. Responses to repetitive application of AMPA remained stable throughout the recording period (up to 15 minutes).

It must however be noted that the 5\(\beta\)-hydroxy isomer of the same steroid, 3\(\alpha\), 5\(\beta\)-THDOC, did not have any neuromodulatory influence on the AMPA receptor responses. The AMPA dose-response relationships in the presence of 1 and 10 \(\mu\)M concentrations of 3\(\alpha\), 5\(\beta\)-THDOC did not yield EC\(_{50}\) values that were significantly different from those of the control neurones (24.27±1.69, 26.83±1.84 and 25.55±1.02 \(\mu\)M for 1 and 10 \(\mu\)M 3\(\alpha\), 5\(\beta\)-THDOC, and control neurones, respectively; \(p>0.05\), Student’s \(t\)-test).
Figure 6.11 AMPA (30 μM) evoked whole cell currents in a control acutely dissociated neurone (A), in the presence of varying concentrations of THDOC (B-D), and following a washout period of 3 minutes (E).
Table 6.5  The effect of THDOC on peak AMPA dose-response relationships of acutely dissociated neurones (n=6, 9 and 15 for 0.1, 1 and 10μM THDOC, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>THDOC (0.1μM)</th>
<th>THDOC (1μM)</th>
<th>THDOC (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (μM)</td>
<td>25.15±1.08</td>
<td>30.92±1.35</td>
<td>34.71±1.53</td>
<td>60.86±2.35</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>-</td>
<td>1.23±0.07</td>
<td>1.46±0.14</td>
<td>2.42±0.10</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.36±0.05</td>
<td>1.42±0.08</td>
<td>1.58±0.09</td>
<td>1.92±0.11</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±0.72</td>
<td>97.96±1.14</td>
<td>97.12±1.88</td>
<td>96.35±2.03</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.6  The effect of THDOC on plateau AMPA dose-response relationships of acutely dissociated neurones (n=6, 9 and 15 for 0.1, 1 and 10μM THDOC, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>THDOC (0.1μM)</th>
<th>THDOC (1μM)</th>
<th>THDOC (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (μM)</td>
<td>2.59±0.24</td>
<td>2.81±0.94</td>
<td>4.36±1.15</td>
<td>6.07±0.65</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>-</td>
<td>1.09±0.03</td>
<td>1.68±0.09</td>
<td>2.35±0.07</td>
</tr>
</tbody>
</table>
Figure 6.12  AMPA dose-response relationships showing whole cell peak current responses to AMPA alone (■) and in presence of 0.1μM THDOC (▼), 1μM THDOC (▼) and 10μM THDOC (▼), recorded from dissociated hippocampal neurones clamped at -40mV.

Figure 6.13  The degree of fade to plateau of control AMPA receptors expressed as the percentage desensitisation in control neurones (dotted line), and control neurones + 10μM THDOC (▼).
6.8 Modulation of AMPA receptor responses by Allopregnanolone and Epiallopregnanolone

The AMPA log.concentration-response curve in the presence of 1 and 10μM allopregnanolone (Figure 6.14), displays a rightward shift of the curve, resulting in an increase of AMPA EC\textsubscript{50} values for peak currents (Table 6.7). The presence of allopregnanolone also resulted in the increase of plateau EC\textsubscript{50} values (Table 6.9), in an analogous manner to THDOC.

The percentage desensitisation of receptor responses, in the presence of 10μM allopregnanolone, was not found to be statistically different from control values; AMPA concentrations of 3.77±0.57 μM and 3.52±0.68 μM, respectively, yielding 50% desensitization.

As allopregnanolone has a similar structure to THDOC (Figure 6.1), it was decided to repeat these experiments, using the 3β-hydroxy isomer of the former neurosteroid (epiallopregnanolone), to investigate the importance of the 3-OH configuration for the modulation of AMPA receptors.

The resulting log.concentration-response curve in the presence of epiallopregnanolone (Figure 6.15), gave rise to greater peak AMPA EC\textsubscript{50} values, which were found to be statistically different from control values (Table 6.8). There was also no apparent change in the maximum response in the presence of epiallopregnanolone, indicating a surmountable inhibition.
The plateau EC\textsubscript{50} values were similarly increased (Table 6.10), in a concentration-dependent manner. Epiallopregnanolone, was found to have an overall smaller inhibitory influence on AMPA receptor responses compared to THDOC and allopregnanolone.

The percentage desensitisation of AMPA receptor responses in the presence of 10 µM epiallopregnanolone was not found to be statistically different from control values (AMPA concentrations of $3.88 \pm 0.69$ µM and $3.57 \pm 0.90$ µM, respectively, yielding 50% desensitization.
Table 6.7  The effect of Allopregnanolone on peak AMPA dose-response relationships of acutely dissociated neurones (n=12 and 15 for 1μM and 10μM neurosteroid, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Allopregnanolone (1μM)</th>
<th>Allopregnanolone (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>24.75±0.44 μM</td>
<td>31.62±1.03 μM</td>
<td>64.53±1.81 μM</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
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<td>&lt;0.01</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>-</td>
<td>1.28±0.07</td>
<td>2.61±0.10</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.37±0.04</td>
<td>1.58±0.10</td>
<td>1.84±0.09</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±0.63</td>
<td>96.56±1.21</td>
<td>93.12±1.75</td>
</tr>
</tbody>
</table>

Table 6.8  The effect of epiallopregnanolone on peak AMPA dose-response relationships of acutely dissociated neurones (n=8 and 10 for 1μM and 10μM neurosteroid, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Epiallopregnanolone (1μM)</th>
<th>Epiallopregnanolone (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>25.59±0.78 μM</td>
<td>27.66±1.22 μM</td>
<td>47.14±2.89 μM</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>-</td>
<td>1.08±0.06</td>
<td>1.84±0.09</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.28±0.06</td>
<td>1.35±0.09</td>
<td>1.74±0.11</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±0.79</td>
<td>97.96±1.07</td>
<td>97.03±1.47</td>
</tr>
</tbody>
</table>
Table 6.9  The effect of Allopregnanolone on plateau AMPA dose-response relationships of acutely dissociated neurones (n=12 and 15 for 1μM and 10μM neurosteroid, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Allopregnanolone (1μM)</th>
<th>Allopregnanolone (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>2.68±0.15 μM</td>
<td>3.07±0.80 μM</td>
<td>4.45±1.37 μM</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>1.15±0.04</td>
<td>1.66±0.12</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.10  The effect of epiallopregnanolone on plateau AMPA dose-response relationships of acutely dissociated neurones (n=8 and 10 for 1μM and 10μM neurosteroid, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>Epiallopregnanolone (1μM)</th>
<th>Epiallopregnanolone (10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>2.77±0.23 μM</td>
<td>2.92±1.20 μM</td>
<td>3.13±1.89 μM</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>&gt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>EC50 ratio</td>
<td>1.06±0.04</td>
<td>1.12±0.05</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.14 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 1 and 10µM Allopregnanolone, recorded from dissociated hippocampal neurones clamped at -40mV.

Figure 6.15 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 10µM Epiallopregnanolone, recorded from dissociated hippocampal neurones clamped at -40mV.
6.9 Comparison of the influence of neurosteroids on the rate of decay of AMPA currents from peak

In order to obtain a further measure of the neurosteroid-induced modification of AMPA desensitization by pregnenolone sulphate (n=7), corticosterone (n=4) and THDOC (n=6), the current decay from peaks of the available electronic data (examples shown in Figure 6.16) were fitted by a single exponential (method described in section 2.1.10).

Pregnenolone sulphate and corticosterone caused small reductions in the $\tau_D$ values of the AMPA current decay which were statistically significant (Student’s $t$-test). THDOC, however, caused a larger reduction in $\tau_D$ (Table 6.11).

Table 6.11 The effect of neurosteroids on AMPA current decay, shown as the rate constant of a single exponential fit ($\tau_D$).

<table>
<thead>
<tr>
<th>Current decay</th>
<th>Control</th>
<th>+ PS (10 μM)</th>
<th>+ CORT (10 μM)</th>
<th>+ THDOC (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_D$ (ms)</td>
<td>48.76±1.08</td>
<td>35.76±1.26</td>
<td>41.34±2.91</td>
<td>32.55±1.53</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
Figure 6.16 AMPA (300μM) evoked whole cell currents in acutely dissociated hippocampal neurones showing the degree of decay of response in the presence of A) pregnenolone sulphate (10 μM) and corticosterone (10 μM), B) THDOC (10 μM).
6.10 Further investigation of the neuromodulatory site/s on the AMPA receptor

a) Use of NBQX

Experiments were carried out in the combined presence of the highest concentration of THDOC utilized in these assays (10 μM) and the competitive AMPA receptor antagonist, NBQX (6 μM), in order to investigate whether these agents act through common or distinct sites. The resultant log.concentration-response curve (Figure 6.17) yielded an increase in the AMPA EC$_{50}$ ratio (Table 6.12), which was found to be statistically significant, compared to experiments with 10μM THDOC alone ($p<0.001$, Student’s $t$-test).

The most comprehensive pharmacological approach in the study of compounds that appear to act competitively is by Schild analysis. However, true antagonism requires parallel dextral displacements of dose-response curves that result in a linear Schild regression with a slope of unity (plotted as log (dr-1) against log antagonist concentration). The neuromodulatory influence of THDOC, although surmountable at higher agonist concentrations, did not give rise to a parallel shift with increasing concentrations and hence did not yield to Schild analysis.

Table 6.12 The effect of combined presence of 10 μM THDOC and 6 μM NBQX on AMPA EC$_{50}$ values, compared to individual exposures.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control + NBQX</th>
<th>Control + THDOC</th>
<th>Control + NBQX and THDOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>113.24±2.03 μM</td>
<td>59.93±2.45 μM</td>
<td>188.39±1.97 μM</td>
</tr>
<tr>
<td>EC$_{50}$ ratio (vs relevant control)</td>
<td>4.61±0.09</td>
<td>2.3±0.15</td>
<td>7.65±0.13</td>
</tr>
</tbody>
</table>
b) Use of cortisol

The use of 10µM cortisol in combination with 10µM THDOC (n=6) was not found to influence the resultant log.concentration-response curve, compared to control neurones in presence of 10µM THDOC alone (EC\textsubscript{50} ratios=2.39±0.16 and 2.42±0.10 (Table 6.5), respectively).

In contrast, the simultaneous exposure of cortisol (10 µM) was found to alter the inhibitory influence of pregnenolone sulphate. The AMPA dose-response relationship (Figure 6.18), showed a smaller increase in the peak AMPA EC\textsubscript{50} value in the combined presence of cortisol and pregnenolone sulphate (10 µM) (43.15±2.92 (n=14), than in presence of pregnenolone sulphate alone (48.30±2.57) (n=7); p<0.05, unpaired Student’s t-test).

The maximum response to AMPA was reduced to a significantly smaller extent (p<0.05) by pregnenolone sulphate in the presence of cortisol than in its absence (reductions to 73.36±1.57% and 66.82±1.44%, respectively).

The presence of cortisol therefore resulted in a small reduction of the inhibitory influence of pregnenolone sulphate.
Figure 6.17 AMPA dose-response relationships showing whole cell peak current responses to AMPA for control (■), in presence of 10 μM THDOC (▼) (n=8), 6 μM NBQX (▼) (n=6) and 10 μM THDOC + 6 μM NBQX (▼) (n=10) recorded from acutely dissociated hippocampal neurones clamped at −40mV.

Figure 6.18 AMPA dose-response relationships showing whole cell peak current responses to AMPA for control (■), in presence of 10 μM cortisol (▼) (n=5), 10 μM pregnenolone sulphate (▼) (n=7) and 10 μM cortisol + 10 μM pregnenolone sulphate (▼) (n=14) recorded from acutely dissociated hippocampal neurones clamped at −40mV.
6.11 Discussion

A number of studies have suggested the existence of multiple steroid recognition sites linked to the GABA$_A$ and NMDA receptors. Some studies have reported biphasic concentration-response curves for steroid potentiation of native (Morrow et al., 1990) or recombinant (Puia et al., 1990) GABA$_A$ receptor function, leading to the suggestion that steroid positive modulators may act through multiple sites or different conformations of the receptor. Studies of steroid modulation of $[^{35}S]r$-butylbicyclophosphorothionate ($^{35}$S-TBPS) and $[^3H]flunitrazepam$ binding to brain membranes have also revealed complex steroid interactions that have been interpreted in terms of multiple binding sites (Majewska et al., 1987; Gee et al., 1988; Park-Chung et al., 1999).

The neurosteroids investigated in this project also appeared to effect a diverse influence on the AMPA receptor response. They will, therefore, be discussed in three distinct groups based on the similarity of their neuromodulatory behaviour:

**Group 1: Inactive steroids**

The six neurosteroids in this group were not found to influence the AMPA receptor response, however, they could be differentiated structurally in terms of the presence or absence of a double bond in either steroid ring A or B (divided into (1a) and (1b), respectively):

a) Cortisol, testosterone and DHEAS

Cortisol, which exerts a potent inhibitory influence on AChR (Garbus et al., 2001), possesses the same core structure as deoxycorticosterone, with the
exception of the presence of three hydroxyl groups at C-11, C-17 and C-21. Testosterone and DHEAS have been reported to be potent GABA$_A$ modulators, although the sulphated and unsulphated steroids (ie. DHEA) are thought to modulate GABA$_A$ receptor function through distinct sites (Park-Chung et al., 1999). The substitution of hydrogen at C-17 position with a hydroxyl or keto group separates the group (1a) steroids from the double-bonded neurosteroids in group 2.

b) pregnanolone, epipregnanolone and 5β-THDOC

The remaining three steroids do not possess double bonds at C5-C6 position and share a 5β-hydroxy configuration. It is believed that this structural configuration may sterically hinder the binding to a postulated modulatory site. This observation was in light of the potent influences of 5α-THDOC and allopregnanolone on AMPA receptor responses, which their 5β-stereoisomers did not display. This can only be accounted for by a difference in the spatial orientation of the steroid A-ring. The chemical structures showing this difference in spatial orientation of THDOC are depicted in figure 6.19.

![Chemical structures of i) 5α- and ii) 5β-THDOC.](image-url)
The inherent inactivity of the group 1 steroids may additionally be despite their binding to tentative modulatory sites or the absence of allosteric binding domains.

**Group 2: Steroids exerting insurmountable inhibition (Pregnenolone sulphate, deoxycorticosterone, corticosterone and progesterone)**

The negative modulatory effects of pregnenolone sulphate on AMPA receptors in cultured hippocampal neurones was first reported by Bowlby (1993). The neurosteroid has a wide range of effects on membrane channels, but the effects are selective and differ for each channel type. At concentrations in the high nanomolar to micromolar range, pregnenolone sulphate has been shown to potentiate NMDA currents (Irwin et al., 1992; Bowlby, 1993; Park-chung et al., 1997), reduce the magnitude of GABA and glycine currents (Wu et al., 1990; Majewska et al., 1990; Shen et al., 2000; Sooksawate and Simmonds, 2001), and inhibit voltage-activated Ca$^{2+}$ channels in hippocampal neurones (Ffrench-Mullen and Spence, 1991).

The present study on the effect of pregnenolone sulphate on the AMPA receptors, in acutely dissociated hippocampal neurones, also suggested an inhibitory role. The resultant log.dose-response curves (Figure 6.4), displayed an increase in the AMPA EC$_{50}$ values with increasing concentrations of the neurosteroid, as well as a depression of the maximum receptor responses which was more evident at the highest pregnenolone sulphate concentration investigated (10μM). The Hill slope was also significantly increased.

The neurosteroid deoxycorticosterone and corticosterone were also found to have similar neuromodulatory influences on the AMPA receptor response. The presence of an OH
group at C-11, however, caused a reduction in the potency of the latter steroid, possibly through an increased hydrophilicity.

Progesterone also reduced the maximal evoked responses, although at least a 20μM concentration of the steroid was required for a statistically significant change in the peak AMPA EC₅₀ values (Figure 6.10).

Further investigations of the desensitization kinetics of the AMPA currents at near maximal agonist concentrations (Table 6.11) revealed that the greater macroscopic desensitization of the receptors in the presence of pregnenolone sulphate and corticosterone are accompanied by faster rates of entry into desensitized states. Because the decay current is thought to reflect, in part, entry into and exit from desensitized states (Jones and Westbrook, 1995), the reduction of τ_D values, together with the stabilisation of the desensitized receptor conformations, may have resulted in the prolongation of the length of time that AMPA receptors spend in the desensitized state. These factors possibly contributed to the potent reductions of the peak amplitude of the AMPA currents observed, as well as the enhanced EC₅₀ values for the plateau responses.

Group 3: Steroids exerting surmountable inhibition (THDOC, allopregnanolone and epiallopregnanolone)

The absence of a C5-C6 double bond resulted in significant differences in the AMPA modulations displayed by this group, possibly through the loss of the rigid planar conformation imposed by the double bonds in the A/B ring of the previous group. All the group 3 neurosteroids exerted a reduction in the AMPA response in a concentration-
dependent manner, which was overcome at high agonist concentrations, displaying a non-parallel rightward shift of the dose-response curves.

The main pathway of progesterone metabolism in the brain is its conversion into dihydroprogesterone and allopregnanolone, which are the two most abundant brain neurosteroids (Cheney et al., 1995; Compagnone and Mellon, 2000; Pinna et al., 2000). THDOC and allopregnanolone which share a common structure with the exception of the functional grouping at the C21 position (Figure 6.1), were found to possess analogous inhibitory properties. In comparison, epiallopregnanolone, which has a 3β-hydroxy configuration displayed a slightly reduced modulation (Figure 6.12).

This contrasts with the neuromodulatory influence of steroids at the GABA_A receptor, which necessitates a 3α- configuration (Simmonds, 1991; Lambert et al., 1995), 3β-configuration being inactive.

None of the neurosteroids in group 3 showed a statistically significant alteration in the extent of macroscopic desensitization behaviour of AMPA receptors, compared to control neurones. A study of the desensitization kinetics of the current responses in the presence of 10 μM THDOC, however, showed that the AMPA receptor responses undergo a slightly faster rate of entry into the desensitized states. As desensitization is a measure of the ratio of peak and plateau amplitudes, the lack of influence on the receptor population desensitization behaviour, overall, indicates that the peak and plateau components of the currents were equally inhibited.

The Hill slopes were increased in the presence of all three neurosteroids, which may be indicative of a greater receptor affinity for double-ligand binding.
**Location of neuromodulatory binding sites**

The application of individual steroids from both groups did not produce any direct response, indicating that the receptor channels were not gated in the absence of AMPA.

As to the possibility of open channel blockade, it must be noted that such antagonisms are strongly voltage-dependent, due to the charge they carry in the transmembrane field. The pregnenolone sulphate and THDOC inhibitions of AMPA-evoked currents were not found to alter the current-voltage relationships, and were reversible upon a short wash-out period, it is therefore unlikely that their binding site/s are located within the receptor channel.

Also, the inability of the intracellular applications of these steroids to alter receptor activity may be indicative of a site of action at the cell surface or alternatively, at the level of the exofacial leaflet of the membrane. It must be noted that due to the solubility problems encountered with steroids, using higher intracellular steroid concentrations would have necessitated the use of large quantities of DMSO, which could subsequently influence other cell functions, therefore limiting the intracellular neurosteroid concentrations which can be realistically achieved.

The studies on cortisol combined with pregnenolone sulphate showed that at a 10μM concentration, the presence of cortisol caused a reduction in the inhibitory influence of pregnenolone sulphate (Figure 6.18), despite cortisol's inability to exert a modulatory effect of its own on AMPA currents (Figure 6.2). The binding of cortisol to the same modulatory sites as pregnenolone sulphate may provide an explanation for its influence.
A steroidal "saturation" effect whereby a second steroid might be prevented from partitioning into an already steroid-laden bilayer, may however offer an alternative explanation. However, if able to penetrate the membrane, it is surmised that other sterols adopt a position in the membrane similar to that of cholesterol (Loura and Priesto, 1997). The presence of substituent OH groups may affect the extent of penetration into the membrane.

The same competitive effect was not seen with cortisol and THDOC, although given the structural and pharmacological differences observed, it seems unlikely that the steroids in group 2 and 3 function at the same receptor site.

Looking at the neuroactive AMPA modulators, overall, the presence of at least two classes of steroid binding sites may account for the different influences of the latter two groups of neurosteroids on both the AMPA dose-response relationships and the receptor desensitization profiles.
Chapter 7

Effect of Membrane Cholesterol on the Modulation of AMPA Currents by Neuroactive Steroids
7.1 Introduction

As discussed in the previous chapter, as well as their binding to cognate intracellular receptors, certain steroids may also act as functional antagonists at the AMPA receptor. There is possibly a structure-activity relationship for the group of neuroactive steroids which exert a modulatory influence on the AMPA receptor response, and if so, this differs considerably from that known for the GABA\textsubscript{A} receptors (Simmonds, 1991; Lambert et al., 1995).

Over the past decade or so, the dogmatic view that all steroids display a non-specific influence on all membranes has been scientifically challenged. Although at relatively high concentrations steroids can affect cell surface influences such as altered fluidity (Whiting et al., 2000), the discovery of molecular interactions of steroids with membrane components, such as protein receptors, has shifted the momentum away from the view of non-specific membrane/steroid interactions, with regards to the physiologically relevant steroid concentrations.

Cholesterol has been found to modulate the action of general and local anaesthetics on AChR (Arias et al., 1990; Raines et al., 1995), and the influence of neurosteroids on the GABA\textsubscript{A} receptor (Bennett and Simmonds, 1996; Sooksawate and Simmonds, 2001).

It was also shown in previous chapters that the alteration of plasma membrane cholesterol levels resulted in a change of AMPA potency. To determine any effects of cholesterol on neuroactive steroid potency independent of an underlying change in AMPA sensitivity, a ratio of EC\textsubscript{50} values for AMPA in the presence and absence of three
of the neuroactive steroids was determined at different cholesterol states. The high concentration of 10μM was selected for pregnenolone sulphate and THDOC, while using a 1μM concentration for deoxycorticosterone. These concentrations were demonstrated to cause a significant shift of the dose-response curve, hence obtaining a clearer response for the cholesterol-enriched and depleted neurones.

The studies carried out in this chapter were aimed to investigate the influence of altered membrane fluidity on the action of neurosteroids, in addition to any possible sterol-sterol interactions. It was also believed that such experiments could lead to a better understanding of the similarities and differences in the neurosteroid structure-activity profiles.
7.2 Effect of the manipulation of plasma membrane cholesterol on the modulatory influence of 10 μM pregnenolone sulphate

Membrane cholesterol in dissociated hippocampal neurones was depleted (65% of control) or enriched (202% of control) by incubation at 31°C for 20 minutes using methyl-β-cyclodextrin and its inclusion complex, as previously detailed in Chapter 3. The exposure of cholesterol depleted and enriched neurones to 10 μM concentrations of pregnenolone sulphate, resulted in both a rightward shift of the AMPA dose-response curve, and a reduction in the maximum response (Figure 7.1), in the same manner as the control neurones in the previous chapter (Figure 6.4).

Depletion of neurones caused an increase in the antagonism of pregnenolone sulphate on peak AMPA currents (EC₅₀ ratio=2.77±0.11, compared to 1.86±0.13 for controls; p<0.01). The maximum response was, however, reduced by pregnenolone sulphate to a smaller extent (83.52±3.47%, Table 7.1), compared with a reduction of 65.72±1.73% by pregnenolone sulphate in controls (Chapter 6).

The effect of the neurosteroid on the plateau responses of depleted neurones was to increase the EC₅₀ (Table 7.2; EC₅₀ ratio of 2.06±0.09), which was greater than that of the control neurones (EC₅₀ ratio=1.64±0.12, Table 6.2).

In conditions of membrane cholesterol enrichment, the degree of the negative modulatory influence of pregnenolone sulphate was more complex. This was manifest as a much reduced peak EC₅₀ ratio for enriched neurones (1.08±0.07 Table 7.1), compared to the values for control neurones (1.86±0.13, Table 6.1), and a greater reduction in
AMPA maximum response by pregnenolone sulphate to 55.24±2.80% (Figure 7.1B), compared with 65.7±2.17% in control neurones.

Pregnenolone sulphate also enhanced the plateau response EC\textsubscript{50} ratio in cholesterol-enriched neurones (ratio=1.10±0.15; \( p<0.05 \), Student's \( t \)-test). This was similar to, but not to the same extent as the modulation of plateau EC\textsubscript{50} ratio in depleted and control neurones.

The change in AMPA receptor desensitization in the presence of pregnenolone sulphate was slightly less in both cholesterol depleted and enriched neurones compared with control neurones. The AMPA concentration causing 50% desensitization in the presence of pregnenolone sulphate in cholesterol depleted neurones was 3.79±0.61 \( \mu \text{M} \) compared to 7.26±0.48 \( \mu \text{M} \) in the absence of pregnenolone sulphate (\( p<0.001 \)). The analogous values in enriched neurones were 1.41±0.77 \( \mu \text{M} \), in the presence of pregnenolone sulphate, and 2.54±0.36 \( \mu \text{M} \) in its absence (\( p<0.001 \)). These data yielded AMPA concentration ratios of 0.52 in depleted neurones and 0.56 in enriched neurones, compared with 0.44 in control neurones (Chapter 6).
Table 7.1  The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 10μM pregnenolone sulphate in acutely dissociated neurones (n=7 and 12, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + PS</th>
<th>Depleted</th>
<th>Depleted + PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC_{50} μM</td>
<td>55.39±1.73</td>
<td>59.72±2.12</td>
<td>9.56±0.85</td>
<td>26.48±1.93</td>
</tr>
<tr>
<td>P coefficient</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(vs relevant control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC_{50} ratio</td>
<td>-</td>
<td>1.08±0.07</td>
<td>-</td>
<td>2.77±0.11</td>
</tr>
<tr>
<td>(vs relevant control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Projected maximum</td>
<td>100±1.13</td>
<td>55.24±2.79</td>
<td>99.42±1.90</td>
<td>83.52±3.47</td>
</tr>
<tr>
<td>response (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P coefficient</td>
<td>-</td>
<td>&lt;0.001</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(vs relevant control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7.2  The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 10μM pregnenolone sulphate in acutely dissociated neurones (n=7 and 12, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + PS</th>
<th>Depleted</th>
<th>Depleted + PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC_{50} (μM)</td>
<td>1.88±0.22</td>
<td>2.07±0.36</td>
<td>4.91±0.29</td>
<td>10.12±0.32</td>
</tr>
<tr>
<td>P coefficient</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(vs relevant control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC_{50} ratio</td>
<td>-</td>
<td>1.10±0.15</td>
<td>-</td>
<td>2.06±0.09</td>
</tr>
<tr>
<td>(vs relevant control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7.1 The effect of membrane cholesterol alterations by on the concentration-response relationship of peak AMPA currents and their modulation by 10⁻¹⁰M pregnenolone sulphate. A) cholesterol depletion. B) cholesterol enrichment.
Chapter 7

7.3 Effect of the manipulation of plasma membrane cholesterol on the modulatory influence of 1 μM deoxycorticosterone

Following cholesterol depletion, the attenuation of peak AMPA responses by 1μM deoxycorticosterone resulted in an increase in the EC_{50} ratio (Table 7.3) (2.96±0.11, compared to 2.42±0.12 for control neurones).

In a similar manner to pregnenolone sulphate, the maximum response to AMPA was reduced to a smaller extent in depleted neurones (Table 7.3), compared to control neurones reported in the previous chapter. The maxima were reduced to 68.93±3.05% in depleted neurones and 53.56±2.54% in control neurones. Depletion of the neurones also increased the inhibitory effect of deoxycorticosterone on the plateau responses seen in control neurones (Chapter 6).

Cholesterol enrichment, once again, decreased the peak EC_{50} ratio for deoxycorticosterone on AMPA responses (Table 7.3), while causing a greater reduction of AMPA maximum response to 40.36±2.66% by deoxycorticosterone (Table 7.3).

The effect of the neurosteroid on the plateau response was also reduced in enriched neurones (Table 7.4), compared to the antagonism seen in control neurones.

The concentration of AMPA causing 50% desensitization in the presence of deoxycorticosterone was 3.92±0.57 μM in cholesterol depleted neurones, and 1.57±0.42 μM for the neurones which had undergone the enrichment process, yielding desensitization ratios of 0.54 and 0.66, respectively, compared to 0.62 for control neurones (discussed in chapter 6).
### Table 7.3  
**The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 1µM deoxycorticosterone in acutely dissociated neurones (n=13 and 8, respectively).**

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + DOC</th>
<th>Depleted</th>
<th>Depleted + DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>54.87±0.93</td>
<td>62.42±1.87</td>
<td>10.09±0.84</td>
<td>29.83±2.05</td>
</tr>
<tr>
<td>P coefficient (vs relevant control)</td>
<td>-</td>
<td>&lt;0.01</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EC₅₀ ratio (vs relevant control)</td>
<td>-</td>
<td>1.14±0.09</td>
<td>-</td>
<td>2.96±0.11</td>
</tr>
<tr>
<td>Projected maximum response (%)</td>
<td>100±1.71</td>
<td>40.36±2.66</td>
<td>99.42±1.47</td>
<td>68.93±3.05</td>
</tr>
<tr>
<td>P coefficient (vs relevant control)</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Table 7.4  
**The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 1µM deoxycorticosterone in acutely dissociated neurones (n=13 and 8, respectively).**

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + DOC</th>
<th>Depleted</th>
<th>Depleted + DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>1.74±0.10</td>
<td>1.89±0.29</td>
<td>5.02±0.27</td>
<td>9.83±0.36</td>
</tr>
<tr>
<td>P coefficient (vs relevant control)</td>
<td>-</td>
<td>&lt;0.05</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EC₅₀ ratio (vs relevant control)</td>
<td>-</td>
<td>1.09±0.07</td>
<td>-</td>
<td>1.96±0.10</td>
</tr>
</tbody>
</table>
Figure 7.2 The effect of membrane cholesterol alterations on the concentration-response relationship of peak AMPA currents and their modulation by 1μM deoxycorticosterone. A) cholesterol depletion. B) cholesterol enrichment.
7.4 Effect of the manipulation of plasma membrane cholesterol on the 
modulatory influence of 10 µM THDOC

The modulation of AMPA responses by THDOC remained surmountable in both 
cholesterol-enriched and depleted neurones (Figure 7.3).

The rightward shift of the peak AMPA dose-response curve by THDOC was found to be 
increased by cholesterol depletion (Table 7.5) (EC$_{50}$ ratio=2.81±0.11, compared to 
2.42±0.10 for control values reported in Chapter 6; p<0.01, Student’s t-test).

THDOC also reduced the plateau AMPA currents in depleted neurones, although not to 
the extent observed in the previous chapter (EC$_{50}$ ratio=2.13±0.09 in depleted neurones, 
and 2.35±0.07 in control neurones; p<0.05, Student’s t-test).

Cholesterol enrichment conversely reduced the modulatory effect of THDOC on peak 
responses to AMPA (EC$_{50}$ ratio=1.69±0.05; p<0.001) (Table 7.5), although this 
reduction was not as great as those observed with pregnenolone sulphate and 
deoxycorticosterone.

The EC$_{50}$ ratio for the plateau currents also reflects a smaller reduction of these 
responses in cholesterol-enriched neurones (EC$_{50}$ ratio=1.76±0.07 (Table 7.6), compared 
with the reduction of these currents by THDOC in control neurones, p<0.001).

In depleted neurones, THDOC decreased the concentration of AMPA at which 50% 
desensitization was obtained (5.22±0.80 µM in presence of THDOC and 7.37±0.65 µM 
in the absence of the steroid; p<0.01, Student’s t-test). In enriched neurones, as in 
control neurones (Chapter 6), THDOC did not affect receptor desensitization (50%
desensitization=2.91±0.77 μM in the presence of THDOC and 2.78±0.62 μM in its absence).

Table 7.5 The effect of membrane cholesterol enrichment and depletion on modulation of peak AMPA currents by 10μM THDOC in acutely dissociated neurones (n=10 and 9, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + THDOC</th>
<th>Depleted</th>
<th>Depleted + THDOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ μM</td>
<td>52.73±1.12</td>
<td>89.69±1.75</td>
<td>9.98±0.79</td>
<td>28.06±1.86</td>
</tr>
<tr>
<td>P coefficient</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EC₅₀ ratio (vs relevant control)</td>
<td>-</td>
<td>1.69±0.05</td>
<td>-</td>
<td>2.81±0.11</td>
</tr>
</tbody>
</table>

Table 7.6 The effect of membrane cholesterol enrichment and depletion on modulation of plateau AMPA currents by 10μM THDOC in acutely dissociated neurones (n=10 and 9, respectively).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + THDOC</th>
<th>Depleted</th>
<th>Depleted + THDOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (μM)</td>
<td>1.84±0.18</td>
<td>3.24±0.50</td>
<td>4.96±0.27</td>
<td>10.57±0.44</td>
</tr>
<tr>
<td>P coefficient</td>
<td>-</td>
<td>&lt;0.01</td>
<td>-</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EC₅₀ ratio (vs relevant control)</td>
<td>-</td>
<td>1.76±0.07</td>
<td>-</td>
<td>2.13±0.09</td>
</tr>
</tbody>
</table>
Figure 7.3  The effect of membrane cholesterol alterations on the concentration-response relationship of peak AMPA currents and their modulation by 10μM THDOC. A) cholesterol depletion. B) cholesterol enrichment.
7.5 Effect of epicholesterol enrichment

Following on from the observations of the influence of membrane cholesterol status on the modulation of AMPA receptors by the neuroactive steroids, the question was presented once again of whether or not such influences were specific to cholesterol. Therefore, for the next stage of the study, it was decided to explore the modulation of AMPA receptors by THDOC and pregnenolone sulphate in conditions of membrane enrichment with the cholesterol analogue, epicholesterol.

Enrichment of neurones with epicholesterol caused changes in the modulatory potency of THDOC and pregnenolone sulphate, in a similar manner to high membrane cholesterol states.

Peak responses to AMPA in epicholesterol-enriched neurones yielded an EC\textsubscript{50} of 48.87±1.39 μM which was increased to 56.92±1.75 μM (n=6) in the presence of 10 μM pregnenolone sulphate (EC\textsubscript{50} ratio= 1.15±0.08; p<0.05). Pregnenolone sulphate (10μM) reduced the AMPA maximum response to 58.18% in epicholesterol-enriched neurones, compared with 65.72% for AMPA alone.

The exposure of epicholesterol-rich neurones to 10 μM THDOC resulted in an EC\textsubscript{50} for peak responses to AMPA of 69.70±1.83 μM (n=6) compared with 50.23±1.46 μM in the absence of THDOC (EC\textsubscript{50} ratio= 1.37±0.06; p<0.01).

Tables 7.7 and 7.8 display the comparison of peak EC\textsubscript{50} values between exposure of pregnenolone sulphate and THDOC, respectively, in control, cholesterol-enriched and epicholesterol-enriched neurones.
In terms of receptor desensitization, pregnenolone sulphate decreased the AMPA concentration yielding 50% desensitization in epicholesterol-enriched neurones (1.31±0.67 μM, compared to 2.96±0.40 μM in epicholesterol-controls; \( p<0.001 \), Student’s t-test), indicating an increased AMPA receptor desensitization. THDOC did not cause a statistically significant change in the AMPA concentration yielding 50% desensitization in epicholesterol-enriched neurones.

Table 7.7  Comparison of the effect of 10 μM pregnenolone sulphate on AMPA peak responses in control, cholesterol-enriched and epicholesterol-enriched neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control + PS</th>
<th>Cholesterol-enriched + PS</th>
<th>Epicholesterol-enriched + PS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>48.30±2.65 μM</td>
<td>59.72±2.12 μM</td>
<td>56.92±1.75 μM</td>
</tr>
<tr>
<td>EC50 ratio for PS (vs relevant control)</td>
<td>1.86±0.13</td>
<td>1.08±0.07</td>
<td>1.15±0.08</td>
</tr>
<tr>
<td>Projected maximum</td>
<td>65.72±1.73%</td>
<td>55.24±2.80%</td>
<td>58.18±2.44%</td>
</tr>
</tbody>
</table>

Table 7.8  Comparison of the effect of 10 μM THDOC on AMPA peak responses in control, cholesterol-enriched and epicholesterol-enriched neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control + THDOC</th>
<th>Cholesterol-enriched + THDOC</th>
<th>Epicholesterol-enriched + THDOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50</td>
<td>61.95±2.35 μM</td>
<td>89.69±1.75 μM</td>
<td>69.70±1.83 μM</td>
</tr>
<tr>
<td>EC50 ratio for THDOC (vs relevant control)</td>
<td>2.42±0.10</td>
<td>1.69±0.05</td>
<td>1.37±0.06</td>
</tr>
</tbody>
</table>
Figure 7.4  The effect of membrane epicholesterol enrichment on the concentration-response relationship for the modulation of peak AMPA currents. A) In the presence of 10μM pregnenolone sulphate. B) In the presence of 10μM THDOC.
7.6 Discussion

Pregnenolone sulphate, deoxycorticosterone and THDOC, caused a greater rightward shift of the AMPA dose-response curves in cholesterol-depleted neurones, compared to controls. Cholesterol enrichment, on the other hand, reduced the EC$_{50}$ ratios for all three neurosteroids studied, with significantly greater reductions in the influence of pregnenolone sulphate and deoxycorticosterone. However, the reductions of AMPA maximum responses by both pregnenolone sulphate and deoxycorticosterone were more pronounced in conditions of membrane enrichment.

Both pregnenolone sulphate and deoxycorticosterone appear to operate additively with the effect of membrane cholesterol changes, thus the further left the AMPA dose-response curve was placed (depletion), the more prominent the rightward shift by these neurosteroids, and the less prominent was the depression of the observed maximum responses. Conversely, when the dose-response curve was placed at a more rightward position, with the maximum already reduced (enrichment), the neurosteroids caused little further rightward movement, but reduced the maxima more markedly.

Considering the data on steroid structure-activity relationship in terms of AMPA modulation, one interesting factor was noted in terms of the similarity of cholesterol structure to the group 2 steroids in this chapter. It was thought possible that an insurmountable antagonism of AMPA receptor response by cholesterol could explain the reduction of AMPA maximum responses observed following cholesterol (and epicholesterol) enrichment or repletion.
Although the antagonism of THDOC was also somewhat potentiated in cholesterol-depleted neurones and the enrichment of neurones caused a small reduction in the potency of THDOC, the concept of an additive effect with cholesterol seems more remote. As this neurosteroid is thought to act at a different site to pregnenolone sulphate and deoxycorticosterone, other possible mechanisms of interaction, such as fluidity changes, may be more likely.

Whatever the mechanism of interaction between cholesterol and the neurosteroids, epicholesterol appeared to substitute fully for cholesterol, once again, with respect to the effect of enrichment.

With regard to receptor desensitization, the enhancement by pregnenolone sulphate and deoxycorticosterone appeared little changed (slightly reduced) in enriched or depleted neurones; simply adding on to the enrichment-induced enhancement of desensitization, or the depletion-induced attenuation of desensitization. The change in the desensitization rate constant by cholesterol (Chapter 4), possibly governing channel entry into doubly-liganded desensitized states, certainly mimics the pregnenolone sulphate and deoxycorticosterone influence.

A notable difference amongst the action of the three neurosteroids was that THDOC did not statistically alter the overall desensitization of control neurones, and those which were cholesterol- and epicholesterol-enriched, but it did increase the desensitization of the cholesterol-depleted neurones.
Given that all the neuroactive steroids investigated in this project were found to have inhibitory influences on the AMPA receptor, it was decided to compare the influence of these steroids to the behaviour of AMPA receptor antagonists with typical competitive and non-competitive influences, in control neurones, as well as in conditions of altered membrane cholesterol levels. It was hoped that any similarities or differences in their alteration of AMPA receptor function could lead to a better understanding of possible common or distinct sites of action on the receptor.
Chapter 8

Effect of Membrane Cholesterol

on the Modulation of AMPA

Currents by Receptor Antagonists
8.1 Introduction

The quinoxalinedione, NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)-quinoxaline), acts as a selective and competitive AMPA receptor antagonist (Sheardown et al., 1990; Namba et al., 1994; Swedberg et al., 1995; Kapus et al., 2000).

2,3-benzodiazepines are a class of orally active compounds that act as non-competitive antagonists at non-NMDA receptors (Rogawski, 1993). These compounds have been shown to antagonize AMPA receptor-mediated responses in hippocampal neurones and show selectivity for AMPARs over kainate receptors (Lerma et al., 1993; Wilding and Huettner, 1995). The muscle relaxant GYKI52466 [1-(4-aminophenyl)-4-methyl-7,8-methyl-enedioxy-5H-2,3-benzodiazepine HCL], which is a better known member of this group, does not appear to interact with the agonist/antagonist site to which glutamate and quinoxalinedione inhibitors bind (Donovan and Rogawski, 1993; Zorumski et al., 1993; Kessler et al., 1996) nor appears to act as a channel blocker (Donovan and Rogawski, 1993), and may rather be considered as an allosteric modulator which down-regulates receptor function (Arai, 2001).

The 2,3-benzodiazepines, also referred to as homophthalazines, cross the blood-brain barrier rapidly, and hence may help protect neurones from the excitotoxic effects of maintained high levels of glutamate, by enhancing desensitization, whilst allowing fast synaptic transmission to proceed with only minimal changes (Palmer and Lodge, 1993).
One member of this group, tofisopam (Grandaxin®), is already licensed for use in Hungary, Japan and France, for the short-term treatment of anxiety disorders, and reported to be devoid of the sedative properties of the conventional benzodiazepines.

In the present study the effects of the antagonists NBQX and GYKI52466 on control, and then subsequently, on cholesterol-enriched and cholesterol-depleted neurones were investigated.
8.2 NBQX Antagonism

NBQX (0.1-500\mu M) caused a concentration-dependent block of the peak responses to 30\mu M AMPA (Figure 8.1).

The IC\textsubscript{50} was calculated as 5.8±0.14\mu M for this preparation. Hence the NBQX concentration used in all subsequent experiments was 6\mu M.

The neuronal responses to applications of the relevant concentrations of AMPA did not return to the original current amplitudes immediately after the removal of NBQX. This is believed to be due to the time interval required for a washout period of antagonist and establishing a new equilibrium.

![Figure 8.1](image)  

Figure 8.1  Peak current responses to 30\mu M AMPA in the presence of varying concentrations of NBQX, recorded from acutely dissociated hippocampal neurones clamped at -40mV (n=9).
8.2.1 Effect of NBQX on AMPA Currents

An example of the currents induced by increasing concentrations of AMPA on a control neurone, prior to and in the presence of 6μM NBQX, displays the attenuation of evoked responses by NBQX which was overcome at high concentrations of the agonist (Figure 8.2). At low concentrations of AMPA, NBQX antagonized both the peak and plateau components in a concentration-dependent manner.

The degree of reduction of the peak currents were typically found to be greater than those of the plateau, and the percentage change of the plateau currents varied to some extent amongst the acutely dissociated hippocampal neurones.

a) Effect of NBQX on peak AMPA EC\textsubscript{50} in control neurones

Figure 8.3 shows the log.dose relationship for AMPA currents in acutely dissociated neurones in presence of 6μM NBQX. There is a rightward shift of the control curve in the presence of this antagonist with an increase in the calculated EC\textsubscript{50} values which was found to be statistically significant (Table 8.1), and yielded an EC\textsubscript{50} ratio of 4.82±1.26. The surmountable antagonism and hence a parallel shift of the AMPA dose-response curve in the presence of NBQX is in line with the proposed mechanism of action of this substance as a competitive antagonist of AMPA receptors.
Figure 8.2 AMPA currents in a control (A) acutely dissociated hippocampal neurone, and in the same neurone, in the presence of 6μM NBQX (B).

Figure 8.3 AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of 6μM NBQX, recorded from dissociated hippocampal neurones clamped at -40mV (n=13).
Table 8.1  The effect of NBQX on peak AMPA dose-response relationships of acutely dissociated neurones. Statistical analysis carried out using Students’s t-test.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>24.66±0.89 µM</td>
<td>119.42±1.74 µM</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.34±0.03</td>
<td>1.42±0.08</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired control)</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

b) Effect of NBQX on plateau AMPA EC$_{50}$ in control neurones

In an analogous manner to the peak currents, the normalized steady-state plateau response showed an increase of the calculated AMPA EC$_{50}$, in the presence of 6µM NBQX, which was found to be statistically significant (Table 8.2).

Table 8.2  The effect of NBQX on plateau AMPA dose-response relationships of acutely dissociated neurones. Statistical analysis carried out using Students’s t-test.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>2.63±0.17 µM</td>
<td>3.89±0.33 µM</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EC$_{50}$ ratio</td>
<td>-</td>
<td>1.46±0.12</td>
</tr>
</tbody>
</table>
8.2.2 Effect of NBQX on the decay from peak to plateau AMPA currents

For investigation of the fade to plateau of AMPA receptor responses in the presence of NBQX, the degree of desensitization of AMPA currents in the control neurone and also upon exposure to the antagonist were analysed as the percentage ratio of steady-state current (Iss) to peak current (Ip) at each agonist concentration (Figure 8.4), in an analogous manner to section 3, chapter 4.

The effect of NBQX antagonism on the percentage desensitization of receptor responses was not found to be statistically significant, compared to control neurones (50% desensitization=3.42±0.29 and 3.98±0.34, respectively, p>0.05).

![Figure 8.4](image)

Figure 8.4 The degree of fade to plateau of control AMPA receptors expressed as the percentage ratio of steady-state plateau current (Iss) to peak current (Ip) of control neurones and in the presence of NBQX.
8.2.3 Effect of the manipulation of plasma membrane cholesterol on NBQX antagonism

Following on from the observation in Chapter 4 that cholesterol reduced the AMPA EC$_{50}$ in acutely dissociated neurones, it was obviously of interest to investigate whether NBQX would exert a similar influence in states of altered membrane cholesterol.

Samples of the acutely dissociated neurones were incubated with methyl-β-cyclodextrin and its cholesterol inclusion complex using the depletion and enrichment protocol reported in Chapter 3. The cholesterol content of the neurones utilized is tabulated below (Table 8.3).

Table 8.3 The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC (enrichment).

<table>
<thead>
<tr>
<th>Cholesterol content</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmole.mg protein$^{-1}$</td>
<td>0.294±0.016</td>
<td>0.582±0.021</td>
<td>0.174±0.024</td>
</tr>
<tr>
<td>Sample number</td>
<td>17</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>198±4.3%</td>
<td>59±2.8%</td>
</tr>
</tbody>
</table>
a) Effect of cholesterol status on antagonism of peak AMPA currents by NBQX
The normalized dose-response curves showing the antagonism of AMPA currents by 6μM NBQX in control, depleted and enriched neurones (Figure 8.5) display a shift to the right without a change in the maximal AMPA response. The AMPA EC\textsubscript{50} value was therefore increased in the presence of NBQX in both cholesterol enriched (Table 8.4) and depleted neurones (Table 8.5).

The shift in EC\textsubscript{50} ratio in the presence of NBQX was calculated at 4.82±1.26, 4.32±2.09 and 4.41±1.87 (p>0.05 in both instances) for control, enriched and depleted neurones respectively. Hence the cholesterol status of the neuronal membrane was not found to influence the antagonism of AMPA currents by NBQX.

b) Effect of cholesterol status on antagonism of plateau AMPA currents by NBQX
The plateau current AMPA EC\textsubscript{50} values for cholesterol depletion in the presence of NBQX was found to be statistically different from the equivalent values in control neurones (P<0.05) (Tables 8.7 and 8.2). The corresponding EC\textsubscript{50} values for cholesterol enrichment were not found to differ from those of the control neurones in the presence of the antagonist (Tables 8.6 and 8.2).

NBQX did not influence the receptor desensitization in cholesterol-depleted and enriched neurones. The AMPA concentration yielding 50% desensitization in the presence of NBQX in cholesterol-depleted neurones was 7.42±0.55 μM, compared to 7.63±0.81 μM in the absence of NBQX. The analogous values in enriched-neurones were 2.27±0.63 in the presence of NBQX, and 2.46±0.39 μM in its absence (p>0.05).
Table 8.4  The effect of membrane cholesterol enrichment on antagonism of peak AMPA currents by NBQX in acutely dissociated neurones (n=10).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ µM</td>
<td>55.34±0.92</td>
<td>239.54±2.56</td>
</tr>
<tr>
<td>P coefficient (vs paired enriched)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>1.79±0.07</td>
<td>1.91±0.10</td>
</tr>
<tr>
<td>P coefficient (vs paired enriched)</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Table 8.5  The effect of membrane cholesterol depletion on antagonism of peak AMPA currents by NBQX in acutely dissociated neurones (n=14).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>9.40±0.57</td>
<td>41.52±2.39</td>
</tr>
<tr>
<td>P coefficient (vs paired depleted)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hill slope</td>
<td>0.98±0.03</td>
<td>1.16±0.08</td>
</tr>
<tr>
<td>P coefficient (vs paired depleted)</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>
Figure 8.5  The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on the concentration-response relationship for the antagonism of AMPA currents by 6μM NBQX. A) cholesterol enrichment. B) cholesterol depletion.
Table 8.6  The effect of membrane cholesterol enrichment on antagonism of plateau AMPA currents by NBQX in acutely dissociated neurones (n=10).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>1.88±0.17 μM</td>
<td>2.63±0.29 μM</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired enriched)</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EC$_{50}$ ratio</td>
<td>-</td>
<td>1.38±0.10</td>
</tr>
</tbody>
</table>

Table 8.7  The effect of membrane cholesterol depletion on antagonism of plateau AMPA currents by NBQX in acutely dissociated neurones (n=14).

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + NBQX</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$</td>
<td>5.10±0.17 μM</td>
<td>9.27±0.32 μM</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired depleted)</td>
<td>-</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EC$_{50}$ ratio</td>
<td>-</td>
<td>1.81±0.09</td>
</tr>
</tbody>
</table>
8.3  GYKI 52466 Antagonism

GYKI 52466 (0.1-300μM) concentration-dependently reduced peak responses to AMPA (Figure 8.6).

The IC\textsubscript{50} for peak currents was calculated as 13.07±0.74μM for this preparation. Hence the GYKI52466 concentration used in all subsequent experiments was 15μM.

A washout period was required for the recovery of AMPA responses following exposure to GYKI 52466.

8.3.1  Effect of GYKI52466 on AMPA EC\textsubscript{50}

Examples of the currents induced by increasing concentrations of AMPA in control neurones, in the presence of 15μM GYKI 52466, are shown in figure 8.7. The apparent differences in the response pattern of neurones are a reduction in peak responses with an enhancement of plateau responses by GYKI 52466.
Figure 8.6 Peak current responses to AMPA in the presence of varying concentrations of GYKI52466, recorded from acutely dissociated hippocampal neurones clamped at –40mV (n=6).

Figure 8.7 The antagonism of AMPA currents in control acutely dissociated neurones (A) in the presence of 15μM GYKI52466 (B).
a) Effect of GYKI52466 on peak AMPA EC<sub>50</sub> in control neurones

Application of 15μM GYKI 52466 resulted in a reduction of the maximum of AMPA dose-response curve indicating that it acts non-competitively (Figure 8.8). This is in agreement with previous results obtained in primary cultures of embryonic (Donevan & Rogawsky, 1993) and neonatal (Zorumski et al., 1993) hippocampal neurones. There was a degree of rightward shift of the AMPA dose-response curve and therefore an increase in the EC<sub>50</sub> for AMPA in the presence of the antagonist (Table 8.8), indicating a decrease in the affinity of AMPA receptors in the presence of GYKI 52466.

b) Effect of GYKI52466 on plateau AMPA EC<sub>50</sub> in control neurones

The small potentiation of plateau responses at low concentrations of AMPA (Figure 8.9), in the presence of 15μM GYKI 52466, was found to lead to a statistically significant reduction in plateau EC<sub>50</sub> values (1.52±0.39 μM compared to 2.70±0.22 μM for control neurones; p<0.01; Student’s t-test), giving an EC<sub>50</sub> ratio of 0.56±0.26.

8.3.2 Effect of GYKI52466 on the decay from peak to plateau AMPA currents

AMPA receptor desensitization was markedly attenuated by 15 μM GYKI 52466. The AMPA concentration causing 50% desensitization was increased by GYKI 52466 to 19.01±0.97 μM, compared with 4.08±0.26 μM in the absence of the antagonist (p<0.0001; Student’s t-test). This yielded an EC50 ratio of 4.66±0.08.
Table 8.8  The effect of GYKI52466 on peak AMPA dose-response relationships of acutely dissociated neurones (n=17). Statistical analysis carried out using Students’s $t$-test.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Control</th>
<th>+ GYKI52466</th>
</tr>
</thead>
<tbody>
<tr>
<td>$EC_{50}$</td>
<td>$23.97 \pm 0.69 \mu M$</td>
<td>$34.01 \pm 1.52 \mu M$</td>
</tr>
<tr>
<td>$P$ coefficient (vs paired control)</td>
<td>-</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>$EC_{50}$ ratio</td>
<td>-</td>
<td>$1.42 \pm 0.07$</td>
</tr>
<tr>
<td>Maximal response (%)</td>
<td>$100 \pm 0.43$</td>
<td>$57.56 \pm 0.88$</td>
</tr>
</tbody>
</table>

Figure 8.8  AMPA dose-response relationships showing whole cell peak current responses to AMPA in presence of $15\mu M$ GYKI 52466, recorded from dissociated hippocampal neurones clamped at -40mV (n=17).
Figure 8.9  AMPA dose-response relationships showing whole cell plateau current responses to AMPA in presence of 15\,\mu M GYKI 52466, recorded from dissociated hippocampal neurones clamped at -40mV (n=17).

Figure 8.10  The degree of fade to plateau of control AMPA receptors expressed as the percentage ratio of steady-state plateau current (Iss) to peak current (Ip) control neurones in the presence of 15\,\mu M GYKI52466 (n=17).
8.3.3 Effect of the manipulation of plasma membrane cholesterol on GYKI 52466 antagonism

In order to further investigate the influence of GYKI 52466 on acutely dissociated neurones, the effect of membrane cholesterol status on this antagonism was studied. Samples of neurones were incubated with methyl-β-cyclodextrin and its cholesterol inclusion complex as previously. The cholesterol content of the neurones utilized is shown in Table 8.9.

a) Effect of cholesterol status on antagonism of peak currents by GYKI52466

GYKI 52466 exposure resulted in the depression of projected maximal AMPA responses in both cholesterol-enriched (47.68±1.27) and depleted (62.19±1.83) neurones (Figure 8.11).

Depletion of neurones resulted in a small potentiation of the GYKI 52466 antagonistic effect (Table 8.11) (EC$_{50}$ ratio=1.77±0.08, compared to 1.47±0.07 for control neurones in the presence of 15μM GYKI52466). Conversely, cholesterol enrichment caused a reduction of GYKI 52466 antagonism (EC$_{50}$ ratio=1.13±0.10) (Table 8.10).
Table 8.9  The incubation of neurone samples at 31°C for 30 minutes with 5mM methyl-β-cyclodextrin (MβC) (depletion) or cholesterol-methyl-β-cyclodextrin complex (Ch-MβC) at a final concentration of 0.15mM cholesterol + 15mM MβC (enrichment).

<table>
<thead>
<tr>
<th>Cholesterol content</th>
<th>Control</th>
<th>Enriched</th>
<th>Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µmole.mg protein⁻¹</td>
<td>0.265±0.012</td>
<td>0.546±0.030</td>
<td>0.163±0.024</td>
</tr>
<tr>
<td>Sample number</td>
<td>9</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>% Control</td>
<td>-</td>
<td>206±5.2%</td>
<td>62±2.8%</td>
</tr>
</tbody>
</table>

Table 8.10  The effect of membrane cholesterol enrichment on antagonism of peak AMPA currents by GYKI52466 in acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + GYKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>55.77±1.45</td>
<td>61.65±2.49</td>
</tr>
<tr>
<td>P coefficient (vs paired enriched)</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.13±0.10</td>
</tr>
</tbody>
</table>

Table 8.11  The effect of membrane cholesterol depletion on antagonism of peak AMPA currents by GYKI52466 in acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + GYKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>9.58±0.55</td>
<td>16.73±1.17</td>
</tr>
<tr>
<td>P coefficient (vs paired depleted)</td>
<td>-</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.77±0.08</td>
</tr>
</tbody>
</table>
Figure 8.11 The effect of membrane cholesterol alterations by methyl-β-cyclodextrin on the concentration-response relationship for the antagonism of AMPA currents by GYKI 52466. A) cholesterol enrichment. B) cholesterol depletion.
Chapter 8

**b) Effect of cholesterol status on antagonism of plateau currents by GYKI52466**

The plateau current AMPA EC$_{50}$ value for cholesterol enrichment and depletion in the presence of GYKI 52466 was found to be statistically different from respective control values (Table 8.12 and 8.13). The plateau EC$_{50}$ ratios for enrichment and depletion values, demonstrate that the effects of GYKI 52466 on plateau currents were less pronounced in cholesterol–enriched neurones. Depletion, however, enhanced the inhibition of plateau responses (Table 8.13), compared to the GYKI 52466 influence on plateau responses of control neurons ($p<0.01$; Student’s t-test).

The AMPA concentration causing 50% desensitization in enriched neurones was $5.33 \pm 0.78$ μM in the presence of GYKI 52466 and $2.77 \pm 0.42$ in its absence, giving a ratio of $1.92 \pm 0.11$. In depleted neurones, the corresponding concentrations were $11.26 \pm 0.84$ in the presence of GYKI 52466 and $7.16 \pm 0.39$ μM in its absence, giving a desensitization ratio of $1.57 \pm 0.09$. Thus, both cholesterol enrichment and depletion diminished the effect of GYKI52466 in attenuating AMPA response desensitisation.
Table 8.12  The effect of membrane cholesterol enrichment on antagonism of plateau AMPA currents by GYKI 52466 in acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Enriched</th>
<th>Enriched + GYKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>1.77±0.26</td>
<td>1.48±0.48</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>0.83±0.07</td>
</tr>
</tbody>
</table>

Table 8.13  The effect of membrane cholesterol depletion on antagonism of plateau AMPA currents by GYKI 52466 in acutely dissociated neurones.

<table>
<thead>
<tr>
<th>Extrapolated values</th>
<th>Depleted</th>
<th>Depleted + GYKI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀ (µM)</td>
<td>4.45±0.33</td>
<td>2.59±0.37</td>
</tr>
<tr>
<td>P coefficient (vs paired control)</td>
<td>-</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>EC₅₀ ratio</td>
<td>-</td>
<td>1.79±0.09</td>
</tr>
</tbody>
</table>
8.4 Discussion

The competitive AMPA antagonist NBQX caused a 4.8-fold shift to the right of the log.
Concentration-response curve for AMPA without depression of the maximum response.
The parallel shift of AMPA concentration-response curves by NBQX is in close
agreement with whole-cell data from cultured hippocampal neurones (Donevan and
Rogawski, 1993; Parsons et al., 1994) and for AMPA receptors expressed in xenopus
oocytes (Randle et al., 1992) and is in line with the proposed action of this substance as
a competitive antagonist of AMPA receptors.

NBQX also did not influence the Hill coefficient of the AMPA dose-response curve and
the percentage decay from peak response to plateau maintained the same dependency on
AMPA concentration in the presence and absence of NBQX.

The non-competitive antagonist GYKI52466 depressed the maximum of the log.
Concentration-response curve to 57.6 ± 0.88 % of control, with a 1.42 fold increase in
the EC$_{50}$ value. The dependency on AMPA concentration of the percentage decay from
peak response to plateau was shifted by GYKI52466 to 2.8-fold higher concentrations of
AMPA but the maximum percentage decay was unchanged by the antagonist.

A distinct modulatory site on AMPA receptors has been described (Zorumski et al.,
1993; Yamada and Turetsky, 1996, Donevan and Rogawsky 1998), at which 2,3-
benzodiazepines such as GYKI 52466 act to block non-competitively AMPA and
kainate currents in hippocampal neurones. However, the observed reduction in the
affinity of AMPA in the presence of GYKI 52466 is not typical of pure non-competitive
antagonism. In the presence of a classic non-competitive antagonist, the agonist becomes
ineffective and the maximum effect on the dose-response curve is decreased without shifting the curve along the concentration axis. The effect is equivalent to a change in the “intrinsic activity” of the agonist without a change in affinity, so that the dose-response curve merely undergoes compression about the ordinate. Deviation from “true” noncompetitive antagonism has also been observed with picrotoxin, the noncompetitive GABA<sub>A</sub> antagonist (Constanti, 1978).

Blockade of AMPA-induced desensitization by GYKI 52466 was initially used to explain the fact that normally strongly desensitized plateau responses (i.e. small steady-state currents) to AMPA were actually potentiated by GYKI52466 (Zorumski et al., 1993). However, electrophysiological studies using rapid perfusion techniques have shown that 2,3-benzodiazepines have no effect on the rate of AMPA receptor desensitization, and possibly act by slowing channel opening upon binding of agonist (Donevan and Rogawsky, 1993; Rammes et al., 1994). It is however difficult to discuss changes in binding behaviour, using the Hill slope of the resultant dose-response curves.

Comparing the insurmountable influences of GYKI52466 and the group 2 neurosteroids in control neurones, the non-competitive antagonist was shown to enhance receptor desensitization, whereas the neurosteroids attenuated this feature (Chapter 6). Within a synaptic network, agents that enhance AMPA desensitization may have advantages in comparison with other AMPA receptor antagonists to the extent that they preferentially block high frequency synaptic signaling and avoid depressing AMPA receptors on interneurones, which would lead to disinhibition and enhanced excitability (Rogawski and Donevan, 1999).
The effect of altered membrane cholesterol states on the nature of AMPA receptor antagonism, although similar results were found in the presence of group 2 neurosteroids and GYKI 52466, conditions of low and high membrane cholesterol were not found to hinder the effects of NBQX, in contrast to THDOC.

With regards to receptor desensitization, both cholesterol enrichment and depletion reduced the effect of GYKI 52466, although membrane lipid changes exerted little influence on the effect by pregnenolone sulphate and deoxycorticosterone on desensitization (Chapter 7). The desensitizations of similarly enriched and depleted neurones were also not affected by the presence of NBQX (Figure 8.4). This was in contrast to the behaviour of THDOC which reduced the desensitization of cholesterol-depleted neurones by 29.2% (Chapter 7).

Given that both THDOC and NBQX, caused a surmountable antagonism of AMPA responses, the difference of behaviour in cholesterol depleted and enriched neurones, make it unlikely that they both exert the same influence on the receptor. There are also differences with respect to the effect on the Hill slope of the dose-response curves, indicating altered binding behaviour of AMPA receptors in the presence of the neurosteroids which cause surmountable inhibition.

Certainly, the differences observed in terms of the desensitization behaviour, for both surmountable and insurmountable neurosteroidal effects, particularly in cholesterol-depleted neurones can be used to argue the binding to separate sites of action on the receptor by the steroids.
Chapter 9

General Discussion
9.1 The influence of membrane cholesterol on AMPA

Just as many membrane enzymes and receptors have been shown to be influenced by membrane cholesterol, so, in the present study, the AMPA receptor responses of acutely dissociated rat hippocampal neurones have also been shown to be influenced by the cholesterol status of the plasma membrane.

Amongst the important physical features of the cholesterol molecule is the planar steroid ring that is relatively conformationally inflexible. These features of its chemical structure govern much of the interaction between cholesterol and the lipid bilayer. For example, when cholesterol is adjacent to a lipid hydrocarbon chain of a phospholipid in a liquid crystalline state, the flat steroid ring system restricts the conformational flexibility of the lipid hydrocarbon chain or, in other words, orders the chain (Stockton and Smith, 1976). The increase in motional order decreases packing defects in the membrane, decreasing passive permeability. No other component of biological membranes affects the membrane in quite this manner (Yeagle, 1993).

A companion concept that is important to this discussion is that most functional proteins undergo conformational changes during their active cycle. If the relevant protein is a membrane protein, such as a receptor, then a requisite conformational change may involve a change in the volume occupied by the protein in the lipid bilayer (Straume and Litman, 1988). If the change in protein conformation involves an increase in the volume occupied by the protein within the membrane, where would that volume come from? It
could be scavenged from the volume elements inherent in packing defects in the membrane.

For transmitter-gated channels, the change in free energy of the ligand bound to its site on the channel as compared to the ligand in solution leads to channel opening. But because ion channels are integral membrane proteins, they can be subject to the influence of allosteric regulators such as the electric field across the membrane and the mechanical stretch of the neuronal membrane. In mechanically activated channels the energy associated with membrane stretch is thought to be transferred to the channel through the cytoskeleton.

The binding of an agonist to the receptor site is an energy-requiring process which involves the conformational change of the AMPA binding site.

The increased rigidity of the membrane in cholesterol-enriched neurones may consequently increase the energy barrier for binding of AMPA to its receptor site. The 3α-hydroxyisomer of cholesterol, epicholesterol, which can change the fluidity of the membrane almost as effectively as cholesterol (Gimple et al., 1997), also showed the same effect as cholesterol in lowering the effect of AMPA. The sterol depletion of membrane may have the opposite influence, hence lowering the energy barrier for binding of agonist.

The study of receptor desensitization under conditions of cholesterol enrichment and depletion, however, suggested that the alterations of the AMPA receptor response, may be a more complex phenomenon, than originally believed, involving a mixture of fluidity effects and possible allosteric modulation.
Although the evoked AMPA currents in this study displayed a slower and a reduced degree of desensitization in cholesterol-depleted neurones, both cholesterol and epicholesterol enrichment resulted in greater desensitization, indicating that the influence on the AMPA receptor is not unique to cholesterol. In hindsight, it would have also been interesting to study the effect of substitution with another cholesterol analogue, such as coprostanol (5β-cholestan-3β-ol) or epicoprostanol (5β-cholestan-3α-ol), as the configuration at the C-5 position was later found to significantly influence the AMPA receptor modulatory capacity.

The speculation of a direct modulation of AMPA receptors by cholesterol, was consequent to the studies on the neurosteroidal influence of group 2 neurosteroids, such as deoxycorticosterone, which possess similar core structures.

### 9.2 Comparison with the effect of membrane cholesterol on other receptors

The ability of a cholesterol analogue to function as a suitable replacement for membrane cholesterol with regards to the modulation of AMPA receptors is in contrast to the receptor systems such as the oxytocin (Gimpl et al., 1997) and the GABA\(_A\) receptors (Sooksawate & Simmonds, 1998). A 3-hydroxyl group in β-configuration has been cited as an important structural feature for the modulation of these receptors.

Cholesterol has also been suggested to be intimately involved in the activation of nAChRs (Fernandez-Ballester et al., 1994, Addona et al., 1998). The presence of interstitial cholesterol binding sites has been proposed for this receptor (Fong and McNamee, 1986), which are exposed during receptor activation, and must be occupied for channel opening to occur (Addona et al., 1998). However, other groups have
reported that other neutral lipids, such as squalene and α-tocopherol, can support nAChR activation, in the absence of cholesterol (Sunshine and McNamee, 1992), suggesting that the ability of such lipids to flip across the membrane bilayer compartments, may be a decisive factor.

9.3 The influence of neurosteroids on AMPA

The problem with a vast number of neurosteroid research methods, in particular in-vivo studies, is that given that all neurosteroids are derived from PREG and given the existence of some reverse metabolic pathways (for example the reverse pathways between the non-sulphated and sulphated forms of PREG and DHEA), the possibility that several neurosteroids might contribute to the effect observed following the administration of a single neurosteroid can not be ruled out. The use of whole cell patch clamp technique on acutely dissociated cells, to investigate the non-genomic influences of neurosteroids on a particular receptor system, eliminated most of the complexities involved in that the study was focused on a rapid and localized effect on a system which is not influenced by a network. The time-scale of investigation and lack of extracellular support also reduced the possibility of steroidal conversions.

The neurosteroids found to influence the AMPA receptor, displayed variation in response in terms of both surmountability of antagonism and influence on receptor desensitization.

The group which caused an insurmountable inhibition, namely, pregnenolone sulphate, deoxycorticosterone, corticosterone and progesterone, reduced the potency of AMPA, while facilitating the rate and degree of receptor desensitization. Conversely, THDOC,
allopregnanolone and epi-allopregnanolone, displayed allosteric antagonisms which were overcome at high agonist concentrations, but did not alter the desensitization of AMPA receptors.

Studies on homomeric AMPA subunit populations, using single-channel recording techniques could shed additional light on the mechanism of cholesterol and neurosteroid modulation of AMPA, in terms of subunit specificity and alterations in channel open/closed time.

Corticosteroids have been reported to rapidly and transiently suppress voltage-dependent ion conductances in hippocampal cells (Kay and Wong, 1987), via a G-protein-coupled mechanism associated with the activation of protein kinase C (Joëls, 2000). It must however be stated that any changes in intracellular Ca\(^{2+}\) levels via the activation of receptors such as the voltage-gated-Ca\(^{2+}\), or Ca\(^{2+}\)-dependent potassium receptors, would not be relevant to systems containing high concentrations of potent sequestering agents such as EGTA. Not to mention that the present studies were carried out under conditions of voltage-clamp, hence making any influence on voltage-gated systems redundant.

Furthermore, the reduction in the amplitude of AMPA responses can not be attributed to a Ca\(^{2+}\) effect as AMPA gates a low conductance cation channel which is permeable to Na\(^{+}\) and K\(^{+}\), but less so to Ca\(^{2+}\) ions. Ca\(^{2+}\) seems to play a role in the desensitization phase of the AMPA response (flip/flop phenomenon) which is secondary and fixed for each set of receptor subunits. As a divalent cation, Ca\(^{2+}\) can influence the AMPA response (Lerma et al., 1994), but that is an extracellular effect as the divalent modulatory site of AMPA receptors is located extracellularly.
It has been suggested that due to the cyclophenanthrone ring structures and hence their lipophilic nature, steroid hormones can intercalate into the bilayer of target cell plasma membranes, potentially altering the fluidity and function of the membrane (Whiting et al., 2000). It must however be noted that the high micromolar concentrations of steroids required for causing membrane perturbation, although used in other laboratories (numerous papers by Farb et al.), may not relevant to this investigation and would almost certainly not be achieved in physiological conditions in-vivo.

Also, the diversity and specificity of the actions of the neurosteroids investigated, on AMPA receptors, as well as other neurotransmitter-gated receptors, make it unlikely that at high nanomolar or low micromolar concentrations, such steroids could act on membrane proteins via a nonspecific membrane-disordering mechanism, like that proposed for short-chain alcohols (Lovinger et al., 1989).

In terms of comparison with the non-competitive antagonist, GYKI 52466, discussed in chapter 8, it is unlikely that the neurosteroids causing insurmountable inhibitions share the same mechanism of action, or indeed binding site, as the classic antagonist has been shown to cause a decrease in the desensitization behaviour of AMPA receptors (Figure 8.10), as opposed to the increased desensitizations observed in the presence of the neurosteroids.

The most simple mechanism to describe the action of non-competitive inhibition is based on the assumption that compounds enter the open-channel, bind to sites within the luminal domain of the receptor, and block ion flux by sterically ‘plugging’ the receptor pore. However, the structure-activity relationship displayed by different neurosteroids,
as well as variability of influence on ligand-gated receptors, is alternatively consistent with the regulation of ion permeation via an allosteric modulation which can be viewed as the structural modification of the receptor channel by the binding of one neurosteroid molecule to its specific high-affinity binding site.

The quantitatively different effects of the group 2 neurosteroids probably reflects their varying extent of penetration in the membrane, resulting in different accessibilities to their site/s of action. Alternatively, differences in the inhibitory potency of different steroids could arise from subtle variations in structure, brought about by different ring substituent groups. The lack of selectivity at the C-3 position with respect to the group 2 neurosteroids might be attributed to the equivalence of the oxygen moieties in the delocalised negative charges of the carboxylate and sulphate esters, which are probably involved in similar binding interactions.

The activity of the neurosteroids which effect surmountable inhibitions of the receptor response, however, was not consistent with a transmembrane site of action.

The structure-activity dependence of the neurosteroids and the steroisomeric specificity at the C-5 position, which determines the planar configuration in the absence of a double bond in ring A/B, differs from those reported for other neurotransmitter-gated receptors. Inspection of the structures of all the steroids found to have AMPA modulatory influence, suggests that certain features are required for activity. These are summarized as follows:
-i) Addition of polar groups at C11 or C17 results in reduction and complete loss of activity, respectively, in steroids which exert insurmountable inhibitions.

-ii) The absence of a C5-C6 double bond, reducing the rigid planar conformation, results in significant pharmacological differences in the modulation of AMPA receptor activity, which may indicate binding to different modulatory sites.

-iii) Both the 3α and 3β configurations of ring A-reduced pregnane steroids show activity on the AMPA evoked currents. The former configuration is however a more potent inhibitor of the AMPA response, supporting a hypothesis that the 3α-steroisomers may act as partial agonists at the same site as the 3β-metabolites.

-iv) A 5α configuration in either 3α- or 3β-reduced metabolite is essential for their modulatory activity, which is overcome at high agonist concentrations.

9.4 Cholesterol interaction with modulators of the AMPA receptor

Membrane cholesterol enrichment from its native level did not influence the antagonist effects of NBQX, and had a small influence on the THDOC modulation of AMPA, yet, the inhibition of receptor responses by GYKI 52466, pregnenolone sulphate and deoxycorticosterone were greatly reduced in states of high membrane cholesterol. Presumed enrichment with epicholesterol had a similar effect, which may have resulted from a similar mechanism to cholesterol.

The mixed influence exerted by pregnenolone sulphate and deoxycorticosterone in cholesterol-enriched neurones suggested a degree of competition between these neurosteroids and cholesterol/epicholesterol, highlighting the possibility of a direct
modulation of AMPA receptors by cholesterol, in addition to or separate from changes in membrane fluidity. This possibly explains the enhanced reduction in the AMPA maximal receptor response, despite the reduced EC$_{50}$ ratios observed in the enriched neurones.

Alternatively, using the membrane fluidity model, discussed in chapters 4 and 5, it can be argued that in cholesterol- and epicholesterol-enriched neurones, the ability of the activated and/or desensitized AMPA receptors to alter their respective conformations is reduced, due to the conditions of reduced fluidity (i.e. the increase in membrane order has an inhibitory effect on receptor protein movement). This was shown to favour AMPA receptor desensitization, possibly through the stabilization of the desensitized state. The additive desensitizing influence of pregnenolone sulphate and deoxycorticosterone, may therefore lead to the presence of very few receptor channels in the activated/activatable state, resulting in the potent reduction of AMPA responses observed.

But these data do not distinguish categorically between generic fluidity changes and those of specific cholesterol-protein interactions. One way to achieve this distinction would be the expression of AMPA receptors in artificial bilayers, in which complete sterol-substitution can be achieved, as described by Sunshine and McNamee (1992), for the nAChR. The disadvantage of this approach is that secondary membrane and cellular influences would be lost, in comparison to studying acutely dissociated neurones, which lends the most relevant interpretation of in-vivo behaviour by single cells in the absence of neuronal network inputs.
It has been observed that pregnenolone sulphate exerts its most prominent effect on the GABA<sub>A</sub> receptors under conditions in which the receptors desensitize (Shen et al., 2000). It would therefore be interesting to examine the influence of pregnenolone sulphate, using a non-desensitizing agonist, such as kainate, or in the presence of cyclothiazide. This exercise would shed some light on the degree of the contribution of altered desensitization on AMPA peak responses, in the presence of the neurosteroid.

Alternatively, deactivation/desensitization kinetics, using single channel studies on homologous AMPA subunits, would allow for detailed kinetic investigations of such receptor modulations. One drawback of excised patch configurations is the loss of neuronal intracellular components which may be involved in the receptor response.

The recovery of AMPA receptor desensitization could occur directly to non-desensitized states, or it may proceed initially via unbinding transitions, with exit from desensitization only occurring after the agonist has dissociated from one or more subunits (Robert et al., 2001). Therefore, although determination of the extent of contribution of receptor desensitization to the reduction of evoked currents may be possible using single channel studies, any quantification would only be relevant to the homogeneous receptor populations, rather than the native neurones with heterogeneous subunit expressions.

The importance of the flip/flop region to the action of cyclothiazide, upon AMPA receptors has been well documented (Partin et al., 1995, 1996), but it is not known if neurosteroid or GYKI52466 antagonism is influenced by alternative splicing or
mutations in the flip/flop region, which would give an indication of the proximity of their sites of action to this region.

Overall, the results indicate that there is clearly an inverse relationship between membrane cholesterol levels and neurosteroid effectiveness. It can be speculated that the presence of excess sterol in the neuronal membrane may induce changes in AMPA receptor properties, as well as or separate to any alterations in membrane fluidity. If the effects of the steroids shown to have AMPA receptor activity, are indeed dependent on the intercalation of these moieties into the membrane, then the cholesterol content, as well as neurosteroid structural properties also determine the influence of the steroids, making such actions more specific than is commonly believed. The steroidal effects on AMPA receptor responses may act independently, or in tandem, with the other potential non-genomic effects, to initiate rapid modulation. The diverse non-genomic mechanisms of neurosteroids may therefore act, to varying extent and at differing times, depending on the developmental and cellular requirements of the cell.

9.5 Sites of cholesterol-neuroactive steroid interaction

Consideration of the difference in the modulatory influence of neuroactive steroids on the AMPA receptor response raised the question of whether the recognition sites are located on the AMPA receptor protein and, if there are multiple sites, are they all accessed by the relatively brief exposures to the neurosteroids employed in the present study? Do the enrichment and depletion of cholesterol affect only the outer leaflet of the plasma membrane bilayer or is the inner leaflet affected as well?
In principle, there are three regions of the membrane through which these neuroactive steroids might modulate membrane proteins: in the bilayer, the lipid-protein interface, or on the protein itself.

It seems unlikely that there are recognition sites for the AMPA-modulatory neurosteroids on the cytoplasmic domains of the AMPA receptor, or indeed on the transmembrane segments that pass through the inner leaflet of the plasma membrane bilayer, since intracellular application of the neuroactive steroids, pregnenolone sulphate and THDOC, did not influence the AMPA response in control neurones, nor change the normal effect of extracellularly applied neurosteroids (Chapter 6). This observation would also suggest that the neuroactive steroids do not cross between the inner and outer leaflets of the membrane to any significant extent. The possible sites of neuroactive steroid interaction postulated are shown in figure 9.1.

The enrichment and depletion of membrane cholesterol will presumably have a greater effect on the cholesterol content of the outer leaflet compared to the inner leaflet of the plasma membrane due to the extracellular contact with the cholesterol carrier systems. However, the achievement of 40% depletion in total cholesterol, when there is evidence that the inner (cytofacial) leaflet contains more than 85% of total cholesterol in synaptic plasma membrane of mouse (Igbavbão et al., 1996; Wood et al., 1999), suggests that the change in membrane cholesterol would have been reflected in the inner leaflet as well.
Chapter 9

It is proposed, therefore, that important recognition sites for the neuroactive steroids are located on the outer half of the transmembrane segments of the AMPA receptor, in particular relevance to group 2 neurosteroids. It cannot be excluded however, that a small component of neuroactive steroid activity involves recognition sites on the extracellular domain of the receptor that may or may not be directly influenced by cholesterol. The behaviour of group 3 neurosteroids, was found to be more consistent with binding to such extracellular domains.
A) A proposed model (not to scale) for the possible modulation sites of neuroactive steroids on receptor proteins (adapted and modified from: Arias, 1997). B) The annular sites are around the outer surface of the protein, while the nonannular sites may be located at the protein/protein interfaces of the subunits.
9.6 In-vivo implications of AMPA receptor modulation

a) Cholesterol implications

In terms of interpretation of the data presented in this thesis, much will depend upon whether the levels of plasma membrane cholesterol in neurones of the CNS are subject to significant fluctuations in vivo. The turnover of brain cholesterol is very low, with half-time of about six months (Lütjohann et al., 1996), but a substantial proportion of this cholesterol is associated with myelin (Jurevics and Morell, 1995). The blood-brain barrier permits only a very small lipoprotein-mediated flux of cholesterol from the plasma, most of the cholesterol being synthesized within the brain (Lütjohann et al., 1996). The result of this is that the cerebrospinal fluid levels of cholesterol are some 40-50-fold lower than the blood plasma cholesterol. Nevertheless, those CNS structures in the hypothalamic area that are weakly protected by the blood-brain barrier may be exposed to higher concentrations of cholesterol and may be vulnerable to fluctuations in the plasma cholesterol. Thus, the fluctuations in the circulation levels of cholesterol could influence AMPA-mediated excitation in certain brain areas.

The human brain has been found to decrease in weight and size during aging as shown in pathological studies (Terry and Katzman, 1983; Skullerud, 1985). Lipid changes in brain during aging have been extensively studied (Svennerholm, et al., 1994) and it has been found that total solids, total lipids and cerebrosides increase into the 20s and decrease in later life. This is in agreement with the findings that the human brain continues to grow until late adolescence and early 20s. Studies with the aim of identifying the CNS membrane lipid changes during physiological aging have found that there is a mild but linear diminution of total solids, major phospholipids, and cholesterol
from as early as 20 until 80 years of age in the brain, particularly in frontal and temporal cortices. After the age of 80, these changes become more pronounced (Svennerholm et al., 1994).

In the CNS, the association between alterations of cholesterol dynamics and pathophysiology is not well understood. Accumulating evidence suggests that naturally low or clinically reduced cholesterol is associated with an increased incidence of suicide and accident which may be mediated by the adverse changes in behaviour and mood (Kaplan et al., 1997). Low serum cholesterol is also associated with the incidence of depressive illness (Morgan et al., 1993). Moreover, drugs which lower serum cholesterol, e.g. pravastatin, result in a significant decrease in erythrocyte and platelet membrane cholesterol in hypercholesterolaemic patients (Lijnen, et al., 1996), and could by analogy, also affect brain functions.

In Alzheimer’s disease (AD), an association with altered cholesterol homeostasis is also recognized, although poorly understood (Wood et al., 1999). A study on membranes prepared from cortical grey matter of AD patients showed that the cholesterol / phospholipid molar ratio was reduced by 30% in the temporal gyrus but unaffected in the cerebellum, which correlated with a reduced width of the bilayer and changes in electron density selectively in the temporal gyrus (Mason et al., 1992). This aberrant pattern could be restored to that found in the membranes from non-AD controls by cholesterol-enrichment of the AD membranes. Additionally, in hippocampus and frontal cortex of AD patients, it has been shown that apolipoprotein E levels are decreased compared with the levels in non-AD tissue (Beffert et al., 1999). It is of interest,
therefore, that apoE-knockout mice show many of the neurotransmitter deficits and loss of memory that are characteristic of AD (Chapman and Michaelson, 1998) and also show a reduced cholesterol content in synaptic plasma membranes with a preferential reduction in the cytofacial leaflet (Igbavboa et al., 1997). Correspondingly, there is evidence that induced depletions of neuronal cholesterol can affect synaptic plasticity and neurodegeneration (Koudinov and Koudinova, 2001; Hering et al., 2003).

It would be interesting to determine whether in vivo fluctuations in neuronal membrane cholesterol would affect the AMPA receptor function to a pathological extent. Although the membrane cholesterol depletions induced in this work may be considered extreme for in-vivo conditions, given that AMPA receptor response is much more pronounced in depleted neurones, it is possible that there may be a link between neurodegeneration due to excitotoxicity, as well as certain childhood epilepsies and EAA receptor hyperexcitability. It may also be possible that the diminution of some idiopathic childhood epilepsies, as the patients enter adolescence and adulthood, can in part be explained by the cholesterol content of the neuronal membrane.

Additionally, as described earlier, the cholesterol levels in the CNS are mainly regulated locally and the circulating levels of cholesterol are not believed to have a major effect on the total CNS cholesterol content. It may however be possible that the use of lipid lowering agents, which are reported to influence the CNS cholesterol levels (Lijnen and Petrov, 1995), could render patients vulnerable to excitotoxic ischaemic events through an enhanced EAA receptor activity.
b) Neurosteroid implications

AMPA receptor antagonists represent important tools for probing AMPA receptor function, and may also have a future therapeutic role in neurological disorders.

The ability of glutamate and aspartate to act as neurotoxins, especially when energy supply is compromised, has given rise to the proposal that selective iGluR antagonists may be employed to prevent the neurodegeneration associated with a variety of disorders including glutamate-mediated neurotoxicity such as ischaemic stroke (Sheardown et al., 1990; Le Peillet et al., 1992).

There are reports that at pharmacologically relevant concentrations, NBQX and GYKI 52466, do not suppress LTP, a cellular mechanism underlying memory formation (Kapus et al., 2000). This suggests that in clinical practice AMPA receptor blockade may have some advantage over NMDA receptor antagonism, which is accompanied by severe memory impairment.

It is gradually becoming clear that the disturbance of neurosteroid homeostasis within the CNS may underlie the development of several neuropathologies (de Kloet, 2000; McEwen, 2002). The emerging understanding of the neuropsychopharmacological properties of neuroactive steroids, involving both rapid and delayed influences, may therefore afford the design of novel therapeutic regimens.

Neurosteroids are also increasingly believed to play a role in the aetiology of depression (Barden et al., 1995). Several studies have shown that allopregnanolone levels in cerebrospinal fluid and plasma are lower in patients diagnosed with unipolar depression than in normal patients, and that these levels, in addition to depression ratings scales,
increased following treatment with fluvoxamine or fluoxetine (Uzunova et al., 1998; Romeo et al., 1998).

There is emerging evidence that AMPA receptors, in particular the edited GluR2 (Ca\(^{2+}\)-permeable) subtype, may play a role in epileptogenesis and in seizure-induced brain damage. Because Ca\(^{2+}\)-permeable AMPA receptors are predominantly expressed in GABAergic interneurones, it is hypothesized that some forms of epilepsy might be caused by reduced GABA inhibition resulting from Ca\(^{2+}\)-permeable AMPA receptor-mediated excitotoxic death of interneurones (Rogawski and Donevan, 1999).

The side-effect and safety profiles of many existing iGluR antagonists, however, do not render these compounds suitable for use as therapeutic agents. Also, most AMPA receptor antagonists which are now available, have short half-lives, low systemic activity and limited blood-barrier penetration.

Studies of seizure episodes in female patients suffering from “catemenial epilepsy”, which is influenced by stages of menstruation have reported a successful treatment protocol by administration of medroxyprogesterone acetate (Mathson et al., 1984). In humans, seizure patterns are altered by factors such as the onset of puberty, pregnancy, and stress, suggesting that there is an underlying hormonal component. Both male and female epileptic patients often exhibit an increase in the severity and frequency of seizures with puberty (Morrell, 1992). Recent studies suggest that both progestins and androgens are involved in the modification of seizures and seizure frequency (Mellon and Griffin, 2002).
Based upon the finding that certain neurosteroids inhibit the agonist response at the AMPA receptor, use can be made of these antagonists to potentiate the antiseizure activity of conventional antiepileptics. They may therefore be used as adjunct treatments in non-responsive cases. It must however be noted that a seizure is the behaviour of a population of neurones, so caution must be applied in interpretation of influence of neurosteroids at the level of dissociated cells.

In recent years, research has also focused on the roles of allopregnanolone because the anxiolytic effects of progesterone have been shown to result mainly from its conversion to this compound (Bitran et al., 1993). In the third trimester of pregnancy, allopregnanolone and pregnanolone levels in plasma average ≥30ng/ml or approximately 100nM (Paul and Purdy, 1992).

THDOC, which exerts a similar influence to allopregnanolone on AMPA receptors, greatly enhances the function of GABA_A receptors and increases inhibitory transmission in various brain regions (Lambert et al., 1995). The antagonism of AMPA receptor function may therefore act in a synergistic fashion to reduce cellular excitability.

As well as modulating voltage-dependent Ca^{2+} receptors (Ffrench-Mullen et al., 1994), the steroid corticosterone has been found to rapidly influence 5HT_3 receptors and stimulate cholinergic input (Joëls et al., 1991, 2000).

Given that brain concentrations of pregnenolone sulphate are in the mid- to high nanomolar range with micromolar concentrations likely in localized cellular compartments (Baulieu and Robel, 1990; Corpechot et al., 1997), it is conceivable that at low micromolar concentrations, the ability of pregnenolone sulphate to reduce voltage-
gated calcium currents (Ffrench-Mullen et al., 1991), enhance NMDA conductance (Wu et al., 1991) and inhibit GABA_A (Majewska et al., 1990; Lambert et al., 1995), with the consequence of excitation of neurones at the post-synaptic level, is counteracted at synapses with fast excitatory conductance by the attenuation of glutamate-gated AMPA responses. The network excitability of neurones, is after all, the combination of various neuronal inputs, enabling different degrees of feed-back modulation.

Future work could involve looking at the AMPA receptor behaviour in the presence of other neuroactive steroids, not pursued in this work due to time restriction and whether the employment of modulatory agents which are known to affect AMPA desensitization, such as cyclothiazide, could alter neurosteroid and cholesterol modulations of AMPA.
9.7 Conclusion

It is postulated that the ligand-induced conformational changes are sensed at the lipid-protein interface, supporting a possible functional linkage between the membrane environment and ligand-mediated ion conductance changes. The results obtained with cholesterol and its 3α-hydroxyl isomer indicated that the influence on the AMPA receptor function is, however, a complex phenomenon, and cannot be attributed to membrane fluidity changes alone. Certainly, the alteration of AMPA receptor desensitizations in different membrane lipid environments suggested a direct influence on the conformational changes responsible for desensitization.

The neurosteroids that were found, so far, to influence the hippocampal AMPA receptor responses, generally behaved as negative modulators of this receptor. The variations in the influence of the neurosteroids, together with the stereoselectivity displayed, indicate the presence of more than one allosteric modulatory site on the AMPA receptor. These binding sites are believed to be separate from those of the typical receptor antagonists. The competition between the steroids causing insurmountable antagonism and membrane sterols also hinted at the possibility of the presence of binding sites for cholesterol on the AMPA receptor.

The steroid modulation of AMPA receptor function, particularly at high local concentrations, would effect consequences for the excitability of neuronal circuits. This suggests an important role for neuroactive steroids, or their analogues, as potential therapeutic tools in the management of seizures, mood disorders such as anxiety, or the prevention of excitotoxic ischaemic events. Alternatively, use can be made of such agents as nootropics, to enhance synaptic strength and LTP.
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222


229


242


