Investigation of the Functional Effects of Two Novel Ampakines in the CNS

Graeme R. Jordan BSc (Hons)

Doctor of Philosophy

The University of Edinburgh

2007
Declaration

In accordance with the regulations of the University of Edinburgh I declare that this dissertation in its entirety is not substantially the same as any that the author has previously submitted for a degree or diploma or other qualification at any other university. The work is solely that of the author, except where and if indicated.

G. R. Jordan
Acknowledgements

Principally, I extend my sincerest gratitude and appreciation to my supervisor Dr Karen Horsburgh and industrial supervisor Dr Brian Henry for their guidance and support throughout the duration of my post-graduate studies, and for their input and saintly patience whilst writing my dissertation. Thanks must also go to the MRC and Organon Research for funding my Industrial Collaborative PhD studentship.

Special thanks must also be given to (1) Professor James McCulloch for his detailed instruction on both brain imaging and structure, for providing technical support for my studies via Fujisawa/ACE, and for his ‘encouragement’ on a day-to-day basis; and (2) Dr Joe Hodgkiss, not only for use of his equipment and lab space, but also for providing good conversation and supplying me with a healthy appreciation of BBC Radio 4 (especially Women’ Hour - I hope I reciprocated by opening his eyes to the lyrical magic that is hip-hop and street slang – Fo’ shizzle).

Finally I would like to thank my fellow students, Andrew Wong, Dr Tom Wishart, Emma Perkins, Oliver Voss and Dr Phil Chen (not really a student, but nevertheless young at heart!) for the beer, banter, gaming and sport (only very occasionally); and last but certainly not least my parents, for their unwavering support and fortitude over the last 3 years.
CHAPTER 1: Introduction

1.1. Glutamate
1.1.1. A historical overview
1.1.2. Glutamate receptor gene families

1.2. AMPA Receptors
1.2.1. AMPA receptor subunit transmembrane topology & stoichiometry
1.2.2. Modifications of AMPA receptor subunits
1.2.3. Ligand-agonist binding core
1.2.4. Channel activation
1.2.5. Receptor desensitisation

1.3. Kainate Receptors

1.4. NMDA Receptors

1.5. Roles of Glutamate in the CNS
1.5.1. Synaptic neurotransmission
1.5.2. Synaptic plasticity
1.5.2.1. Long-term-potentiation (LTP)
1.5.2.2. The role of AMPA receptor regulation in LTP
1.5.3. Excitotoxicity

1.6. Pharmacological Compounds Targeting the AMPA Receptor
1.6.1. AMPA receptor agonists
1.6.2. Competitive AMPA receptor antagonists
1.6.3. Non-competitive AMPA receptor antagonists
1.6.4. AMPA receptor potentiators
1.6.4.1. Plant lectins
1.6.4.2. Pyrrolidinones
1.6.4.3. Thiazides
1.6.4.4. Biarylpropylsulfonamides 35
1.6.4.5. Non-specific AMPA receptor potentiators 35
1.6.4.6. Benzylopiiperidines / Benzoylpyrrolidines 36
1.6.4.7. The Ampakines Org 26576 and Org 24448 37
1.7. The Clinical Utility of AMPA Receptor Potentiators 37
1.8. AMPA Receptor Distribution in the CNS 39
1.9. Imaging the Effects of Drugs in the CNS 41
1.10. Thesis Aims 43

CHAPTER 2: Materials and Methods 45

2.1. Animals 45
2.2. $[^{14}\text{C}]$-2-Deoxyglucose Autoradiography 45
  2.2.1. Theory 45
  2.2.2. $[^{14}\text{C}]$-2-deoxyglucose autoradiography modifications for mouse 48
  2.2.3. $[^{14}\text{C}]$-2-deoxyglucose autoradiography 49
  2.2.4. Densitometric analysis of $[^{14}\text{C}]$-2-deoxyglucose autoradiograms 50
  2.2.5. Statistical analysis of LCG data 51
2.3. Preparation Stages for Investigation of Protein Changes 51
  2.3.1. Saline perfusion 51
  2.3.2. Micro-dissection and tissue preservation 52
  2.3.3. Section preparation and collection 52
2.4. Immunohistochemistry 53
  2.4.1. Theory 53
  2.4.2. General procedure 53
2.5. Western Blot Analysis of Protein Levels 55
  2.5.1. Tissue homogenisation 55
  2.5.2. BCA assay and protein content determination 55
  2.5.3. SDS-PAGE 56
  2.5.4. Western blotting 58
  2.5.5. Coomassie blue staining 59
  2.5.6. Western blot quantification and statistical analysis 61
2.6. Extracellular Electrophysiology

2.6.1 Solution preparation
2.6.2 Acute hippocampal slice preparation
2.6.3 Recording electrode preparation
2.6.4 Electrophysiological recording
2.6.5 Data acquisition and analysis

CHAPTER 3: Characterisation and validation of an in vivo semi-quantitative model of [14C]-2-deoxyglucose autoradiography in the C57Bl/6J mouse

3.1. Introduction

3.1.1. Aims

3.2. Material and Methods

3.2.1. Animals
3.2.2. Drug administration and the [14C]-2-deoxyglucose procedure
3.2.3. Statistical analysis

3.3. Results

3.3.1. [14C]-2-deoxyglucose technique in mouse: control treatment
3.3.1.1. Behavioural and baseline physiological parameters
3.3.1.2. LCGU and comparison with Kelly et al. (2002)
3.3.2. Validation and investigation with MK-801 and CX516
3.3.2.1. Behavioural and baseline physiological parameters
3.3.2.2. Effect of the NMDA receptor antagonist MK-801 on LCGU
3.3.2.3. Effect of the prototypic Ampakine CX516 on LCGU
3.3.3. Pilot study of Org 26576 and Org 24448 on functional activity
3.3.3.1. Behavioural and baseline physiological parameters
3.3.3.2. Regional alterations in LCGU

3.4. Discussion

3.4.1. Justification for and establishing a semi-quantitative model of [14C]-2-deoxyglucose in the mouse
3.4.2. Model validation with MK-801 and investigation with CX-516
3.4.3. Org 24448 and Org 26576 pilot study
CHAPTER 4: Investigation into the effects of acute administration of the novel ampakines Org 26576 and Org 24448 on functional activity in the murine cerebrum

4.1. Introduction

4.1.1. Aims

4.2. Materials and Methods

4.2.1. Animals

4.2.2. Drug preparation, administration and the $[^{14}\text{C}]-2$-deoxyglucose autoradiographic procedure

4.2.3. Statistical analysis

4.3. Results

4.3.1. Org 26576 and Org 24448 dose-response relationship

4.3.1.1. Effect of Org 26576 and Org 24448 on behavioural and baseline physiological parameters

4.3.1.2. Effects of Org 26576 and Org 24448 on LCGU

4.3.2. AMPA receptor antagonist pre-treated mice

4.3.2.1. Effects of the NBQX on behavioural and baseline physiological parameters

4.3.2.2. Effects of NBQX pre-treatment on LCGU

4.4. Discussion

4.4.1. Org 26576 and Org 24448 produce specific effects on LCGU

4.4.2. AMPA receptor distribution

4.4.3. The effects of Org 26576 and Org 24448 on LCGU are AMPA receptor mediated

4.4.4. Functional implications

4.4.5. Summary
CHAPTER 5: Investigation into the effects of chronic administration of the novel Ampakines Org 26576 and Org 24448 on functional activity, neurogenesis and receptor/signalling alterations in the murine cerebrum

5.1. Introduction

5.1.1. Aims

5.2. Materials and Methods

5.2.1. Chronic drug administration

5.2.2. [\textsuperscript{14}C]-2-deoxyglucose autoradiographic procedure

5.2.3. BrdU administration and immunohistochemistry

5.2.4. Quantification of BrdU positive nuclei

5.2.5. Protein analysis

5.2.6. Statistical analysis

5.3. Results

5.3.1. Functional consequences of chronic administration of Org 26576 or Org 24448

5.3.1.1. Effect of Org 26576 and Org 24448 administration on behavioural and baseline physiological parameters

5.3.1.2. Effects of 7 Day administration of Org 26576 (1mg/kg) and Org 24448 (10mg/kg) on LCGU

5.3.1.3. Effects of 28 Day administration of Org 26576 (1mg/kg) or Org 24448 (10mg/kg) on LCGU

5.3.2. Structural consequences of chronic administration of Org 26576 and Org 24448

5.3.2.1. BrdU immunostaining and quantification

5.3.2.2. Hippocampal protein levels

5.4. Discussion

5.4.1. Effects of chronic administration of Org 26576 and Org 24448 on functional activity in the mouse cerebrum

5.4.2. The mesocorticolimbic system

5.4.3. Functional effects and current theories

5.4.4. Structural effects and protein levels implicated in Ampakine function
CHAPTER 6: Modulation of AMPA Receptor Kinetics by Org 26576 and Org 24448 Influences Synaptic Plasticity in the Murine Hippocampus

6.1. Introduction

6.1.1. Aims

6.2. Materials and Methods

6.2.1. Animals and slice preparation

6.2.2. Electrophysiological recordings

6.2.3. Stimulation protocols

6.3. Results

6.3.1. Effects of the Ampakine CX516 on fEPSP kinetics

6.3.2. Effects of the novel Ampakines Org 26576 and Org 24448 on baseline fEPSP responses in acute hippocampal slices

6.3.3. Stable LTP induced by high-frequency-stimulation (HFS)

6.3.4. Effects of ‘physiologically relevant’ concentrations of Org 26576 and Org 24448 on LTP

6.3.5. Effects of EC_{50} concentrations of Org 26576 and Org 24448 on LTP

6.4. Discussion

6.4.1. CX516, Org 26576 and Org 24448 modify extracellular field excitatory-post-synaptic-potentials

6.4.2. Cognition and synaptic plasticity

6.4.2.1. LTP

6.4.2.2. Org 26576 and Org 24448 bidirectional modify LTP

6.4.3. Future experimental approaches

6.4.4. Summary

CHAPTER 7: General Discussion

7.1. Summary of findings
Abstract

The ionotropic glutamate AMPA ((R,S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor mediates the majority of excitatory transmission in the CNS. AMPA receptors play a crucial role in both basal neurotransmission and synaptic plasticity events (such as long-term potentiation, LTP). Compounds that ‘potentiate’ AMPA receptor function (‘Ampakines’) are known to positively modulate glutamatergic AMPA receptor-gated currents, by slowing the deactivation and desensitisation rate of the receptors, in the presence of the endogenous agonist glutamate. Ampakines have been shown to facilitate LTP induction, improve cognition, and as such have potential in the treatment of conditions such as depression and psychoses (schizophrenia). The main aim of this thesis was to investigate the functional actions of two novel Ampakines, Org 26576 and Org 24448, in the mouse brain. The studies described in this thesis were designed to address this and are outlined as follows:

1. Characterisation and validation of an in vivo semi-quantitative model of $[^{14}\text{C}]$-2-deoxyglucose autoradiography in the C57Bl/6J mouse

The first study sought to develop and characterise a model of $[^{14}\text{C}]$-2-deoxyglucose autoradiography, to allow measurement of regional alterations in local cerebral glucose use (LCGU) in the mouse CNS. Following intraperitoneal injection of $[^{14}\text{C}]$-2-deoxyglucose in C57Bl/6J mice, the radiolabelled brains were sectioned and exposed to x-ray film. The resultant autoradiograms were semi-quantitatively analysed for relative optical densities in predetermined regions of interest. The baseline LCGU values in different brain regions were found to be consistent with previously published data. The model was also able to replicate the effects of a well-characterised compound, the NMDA receptor antagonist MK-801 (0.5 mg/kg), in respect to functional cerebral changes. Characteristic effects such as prominent increases in LCGU in the limbic system, and decreases in the somatosensory cortex were reproduced in the model. Thus the semi-quantitative $[^{14}\text{C}]$-2-deoxyglucose model was reproducible and accurate and thus could be further used to investigate the effects of the novel Ampakines, Org 26576 and Org 24448, on cerebral function.
2. Investigation into the effects of acute administration of the novel Ampakines Org 26576 and Org 24448 on functional activity in the murine cerebrum

Following the establishment of the methodology, regional alterations in LCGU in response to the Ampakines Org 26576 and Org 24448 were investigated using $[^{14}\text{C}]$-2-deoxyglucose autoradiography. Both Org 26576 and Org 24448 produced regionally selective, dose-dependent increases in LCGU in the mouse cerebrum when administered acutely (~1 hr). The compounds displayed similar yet functionally distinct profiles of activation, the highest levels of activation occurred in areas of the limbic system (hippocampus), sensory systems, and various nuclei (raphe nucleus). Their effects were blocked by pre-administration of the potent selective AMPA receptor antagonist, NBQX (10 mg/kg), which itself had minimal effects on LCGU. These data provide an anatomical basis for the cerebral activation induced by these compounds, which are directly AMPA receptor mediated. Areas activated also closely correlated with brain regions implicated in various psychiatric conditions, and as such is suggestive of a potential therapeutic benefit of these compounds in conditions such as depression and schizophrenia.

3. Investigation into the effects of chronic administration of the novel Ampakines Org 26576 and Org 24448 on functional activity, neurogenesis and receptor/signalling alterations in the murine cerebrum

Following the demonstration that acute administration of Org 26576 and Org 24448 displayed regionally selective and dose-dependent alterations in LCGU, the effect of chronic administration of the Ampakines Org 26576 and Org 24448 on regional functional alterations ($[^{14}\text{C}]$-2-deoxyglucose autoradiography), neurogenesis (BrdU labelling), and proteins levels (GluR, MAPK, LynK and CREB) (Western blot analysis) were investigated. Chronic administration (7 and 28 days) of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) induced functional cerebral increases in the mouse cerebrum particularly in areas of the mesocorticolimbic system, which were not only rapid in onset, with significant effects visible after 7 days administration; but importantly were also persistent and long lasting. Chronic administration of the
compounds had no significant effect on the level of neurogenesis or on the levels AMPA receptor subunits (GluR1,2,3), and signalling pathways (MAPK/LynK-CREB pathway), implicated in AMPA/Ampakine signalling, in the murine hippocampus. These data show that the Ampakines Org 26576 and Org 24448 when administered chronically can potentiate complex neural networks intimately associated with disease states, the effects of which are maintained over prolonged periods. There was no evidence that this involved an effect on neurogenesis or the MAPK/LynK-CREB signalling pathway.

4. Modulation of AMPA receptor kinetics by Org 26576 and Org 24448 influences synaptic plasticity in the murine hippocampus

The ability of Org 26576 and Org 24448 to modify baseline kinetic properties of AMPA receptors and a paradigm of synaptic plasticity, LTP, in the mouse hippocampus was investigated using electrophysiology. Both Org 26576 and Org 24448 produced dose-dependant increases in fEPSP amplitude without affecting the half-width of responses, in acute hippocampal slices. Concentrations of both compounds, equating to functionally active levels witnessed in vivo, potentiated a stable form of LTP; whilst higher EC50 concentrations prevented the maintenance of LTP. These results are suggestive that Org 26576 and Org 24448 are effective in boosting the neural correlate of cognition, LTP, and may have potential in treating cognitive deficits, for example those associated with depression, schizophrenia or Alzheimer’s disease.

The data presented in this thesis illustrate that the novel Ampakines Org 26576 and Org 24448 centrally modulate brain regions and circuitry intimately associated with conditions such as depression and schizophrenia (psychoses), with effects that are rapid in onset and persistent over chronic periods of administration. Specifically targeting the glutamatergic system through the use of these compounds may provide an innovative approach to treat various conditions that may be partly due to a compromise of normal excitatory glutamatergic neurotransmission.
Chapter 1

Introduction
1.1. Glutamate

1.1.1. A historical overview
Glutamate is the primary excitatory neurotransmitter in the mammalian brain, with approximately 60-80% of all neurons receiving a glutamatergic input. L-glutamate was first purported to be a synaptic transmitter in the brain by Hayashi in 1954, who discovered that injection of glutamate via an intracerebroventricular or intracarotid route produced convulsions in both dogs and monkeys (Hayashi, 1952 & 1954). However, for many years its role as a central transmitter was widely contested. It was argued by its detractors that not only was it present at too high a level in the brain and its function was not confined to a limited number of synaptic pathways, but most importantly it did not fit the stringent and specific criteria at the time used to validate a substance, such as acetylcholine, as a transmitter.

General acceptance of its role as a neurotransmitter was not achieved for a further 20 years, and was mainly due in part to the advent of advanced electrophysiological recording techniques and the discovery of specific glutamate antagonists; both of which allowed the repudiation of all the theoretical obstacles for its role as a transmitter. Initial experiments by Watkins, Curtis and colleagues demonstrated that experiments with glutamate, and both naturally occurring and artificially synthesised analogues such as N-methyl-D-aspartate (NMDA), quisqualic acid and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA); resulted in dramatic depolarisation of neurones with what appeared to be sub-type specific responses. These experiments in turn led to the proposal that a receptor binding site existed for glutamate and its analogues (Curtis & Watkins 1960; 1965; Krogsgaard-Larsen et al., 1960). At the same time, attention was also being focused on the mechanisms of cellular uptake and release of glutamate from nerve terminals (Curtis & Johnston, 1974), which further established reasonable ‘logistics’ for the role of glutamate in synaptic transmission (Watkins, 1972).

The first hint of the existence of multiple excitatory amino acid receptors was provided by Hugh McLennan and colleagues when they compared the actions of DL-homocysteate with glutamate in different regions of the thalamus. They ascribed the
regional differences observed in the relative potencies to the possibility of there being more than a single glutamate receptor (McLennan et al., 1968; Watkins, 2006). Further to this, the Curtis/Johnston team showed that glutamate analogues such as N-methyl-D-aspartate (NMDA), quisqualate, and kainic acid demonstrated varying degrees of potency on a subset of neurones in the spinal cord (McCulloch et al., 1974); further reinforcing the idea of the existence of distinct glutamate receptor subsets.

Further essential evidence was provided by studies carried out at Bristol by Jeffrey Watkins, Tim Biscoe and Richard Evans. Low concentrations of magnesium (0.1-0.2 mM) added to traditional frog Ringer solution reduced evoked synaptic activity in response to a specific subset of amino acids. NMDA-responses were completely abolished, L-glutamate responses were reduced intermediately, whereas kainite and quisqualic acid responses remained virtually unchanged. Work with other compounds such as HA-966 and diaminopimelic acid (DAP) replicated the work with Mg\(^{2+}\) ions (Evans et al., 1979). These three compounds seemed to be acting at distinct sites to produce near identical effects, further reinforcing the idea of different receptors for NMDA and kainite; ultimately leading to an initial classification of receptors into NMDA and non-NMDA (Watkins & Evans, 1981). Non-NMDA receptors were further subdivided into quisqualate and kainite, due to the discovery of kainite-specific effect in dorsal root C-fibres.

With the advent of specific receptor antagonists, especially D-2-amino-5-phosphonopentanoate (D-AP5) (Davies et al., 1982; Evans et al., 1982; Perkins et al., 1982), further supportive evidence of distinct glutamate receptor in synaptic transmission was gathered in the 1980s. Two additional key studies led to additional large changes in the glutamate field. Firstly, it was observed that AMPA was a more potent, selective, quisqualate-like agonist (Krogsgaard-Larsen et al., 1980; Honore et al., 1982). And secondly, L-glutamate and its analogues, specifically quisqualate, were observed to be able to stimulate phosphatidylinositol hydrolysis and intracellular Ca\(^{2+}\) mobilisation (Sladeczek et al., 1985; Nicoletti et al., 1986). This led not only to the renaming of non-NMDA receptors to AMPA and kainate
(Collingridge and Lester, 1989), but also gave rise to a new class of receptors that were functionally coupled to G-proteins and second messenger systems, subsequently termed the metabotropic glutamate receptors (Watkins, 2000).

Fig 1.1. Diagrammatic representation of the major glutamate receptors at an excitatory glutamatergic synapse

Currently therefore, glutamate receptors can be split into two distinct groups: the slow acting metabotropic glutamate receptors (mGluRs), which are coupled to membrane-bound G-proteins and mediate function through various downstream intracellular secondary messenger systems; and the fast-acting ionotrophic glutamate receptors (iGluRs) which mediate function via an integrated cationic ion channel which primarily gates Na\(^+\) influx but also Ca\(^{2+}\) and K\(^+\) (efflux) (Fig 1.2.).
1.1.2. Glutamate receptor gene families

With the advent of advanced molecular biology and the application of cDNA cloning techniques, glutamate receptors were cloned and expressed in recombinant systems in the late 1980’s. Expression cloning in *Xenopus* oocytes revealed the cDNA for the first glutamate receptor subunit to be identified, which in time was determined to be the AMPA receptor subunit GluR1 (Hollmann and Heinemann, 1989). Based on homology cloning for this identified GluR1 subunit the other GluR AMPA subunits were quickly identified: GluR2, GluR3 and GluR4 (Keinanen *et al*., 1990). In total it emerged that NMDA, AMPA, and kainate receptor subunits were encoded by at least 6 gene families: one family for AMPA receptors, two for kainate, and three for NMDA (reviewed Dingledine *et al*., 1999). Within these outlined families there is considerable preserved homology, ~65-75%. However between separate families there is only 40-50% sequence homology; this nonetheless suggests a common evolutionary origin for all the ionotropic glutamate receptor subtypes. Orphan subunits, δ1 and δ2, have also been identified (Lomeli *et al*., 1993). These are distinct structural relatives to the glutamate receptor subtypes sharing only 18-25% amino-acid identity. They do not form functional channels and do not modify the function of other subunit combinations, and are still poorly characterised as their functional activity and ligand-binding properties have as yet defied analysis (reviewed Dingledine *et al*., 1999).

1.2. AMPA Receptors

AMPA receptors are found in the majority of excitatory synapses and function to mediate fast excitatory neurotransmission in the CNS. Their rapid kinetic properties make them ideally suited for this task. Primarily responsible for gating Na⁺, they generally display an inherent low permeability to Ca²⁺, ensuring that glutamate-activated ionic currents mediated by these channels do not carry sufficient Ca²⁺ ions into cells to initiate biochemical or down-stream processes triggered by increases in intracellular calcium levels (Seeburg, 1993).
Fig 1.2. The diverse molecular gene families of the glutamate receptors

An overview of the current classification of ionotropic and metabotropic glutamate receptors, their known subunits, and primary signalling mechanisms. Glutamate activates both cation-permeable ionotropic receptors and metabotropic receptors coupled via G-proteins to activate phospholipase C (PLC) or inhibit adenylate cyclase (AC) activity. Group I receptors also also associated with Na⁺ and K⁺ channels (adapted from Dingledine, 1999 and Kew & Kemp, 2005).
1.2.1. AMPA receptor subunit transmembrane topology & stoichiometry

When setting out to determine the transmembrane topology of the glutamate receptors, it was initially thought the topology would resemble that of the 4 transmembrane model of the nicotinic acetylcholine receptors. In addition to this it was further discovered that their N-terminal and agonist-binding domains shared amino acid sequence homology with bacterial periplasmic-binding proteins, and were thus likely to have a similar structure. However, against all initial expectations the structure elucidated bore a striking similarity to that of K⁺ channels, in that it had a very similar re-entrant (p)loop. The now widely accepted topology for the AMPA receptor subunit, which ranges in size from 102 to 108kDa, is shown in Figure 1.3. There are 4 subunits in all, GluR1-4, which share 68-74% sequence identity at the protein level. Each subunit consists of 3 transmembrane domains, M1, M3 and M4, and a cytoplasm-facing re-entrant membrane loop, M2. This M2 region is thought to line the complete receptor ion pore, and also contains the ‘Q/R’ editing site, which controls key permeation properties of the ion channel. The N-terminal region (400 amino-acids), a major determinant of subtype-specific assembly within the iGluR gene families, is located extracellularly; whilst the C-terminal region (50-100 amino-acids in length), which interacts with numerous cytoskeletal proteins and is important for receptor trafficking, is found intracellularly. Notably, the M3-M4 region occurs extracellularly as a loop, which is important as it determines the desensitising properties of the receptor (Fletcher and Lodge, 1996). The M3-M4 loop also contains two key receptor subunit-editing sites the ‘R/G’ site and the ‘flip/flop’ determinant region. The S1 and S2 domains, located on the N-terminal and M3-M4 loop respectively, are globular glutamate-binding domains that in native receptors exist as a bilobular structure with a cleft available for ligand binding (Armstrong et al., 1998).
Fig 1.3. Transmembrane topology of the AMPA receptor subunit

Transmembrane domains M1, M3, M4, and a cytoplasm facing re-entrant loop M2, with 'Q/R' editing site. The extracellular M3-M4 loop contains the 'R/G' editing site and the 'flip' / 'flop' splice variant site, and S2, which with S1 (N-terminus) forms a bilobular ligand binding domain (adapted from Bennett and Dingledine, 1995).
The individual subunits, outlined above, were thought to form functional AMPA receptors as tetrameric complexes, comprised from pairs of identical heteromeric dimers. This was confirmed by elegant electrophysiological studies in the late 1990s (Rosenmund et al., 1998). However, AMPA receptor subunits (1-4) also have the ability to form homomeric channels. Homomeric assemblies of GluR1/3/4 are permeable to Na\(^+\), K\(^+\) and Ca\(^{2+}\), but the inclusion of GluR2 in heteromeric assemblies imparts the Ca\(^{2+}\) impermeability normally associated with the AMPA receptor. Indeed, the exact distribution of GluR1-4 is varied throughout the CNS; in the CA1 pyramidal cells for example AMPA receptors most commonly occur as paired dimers of GluR1/2 subunits and GluR2/3 subunits, but in the cerebellum and thalamus AMPA receptors are commonly composed of GluR4 subunits in combination with GluR2 (Wenthold et al., 1996). There is also evidence for the coexistence of AMPA receptors of varying subunit combinations in the same neurones (Dingledine et al., 1999). This will be of importance later when considering the actions of AMPA modulating compounds in selective areas of the CNS. AMPA receptors are also abundantly expressed in astrocytes, microglia and oligodendrocytes (Janssens and Lesage, 2001).

### 1.2.2. Modifications of AMPA receptor subunits

Ionotropic glutamate receptor subunits are subject to two main forms of post-translation modification, specifically alternative splicing and RNA editing, to which the high structural and functional diversity of AMPA subunits can be attributed.

All four AMPA subunits GluR1-4 can occur as two alternatively spliced variants ‘flip’ or ‘flop’, encoded by Exons 14 and 15 (in GluR2) in a 38 amino-acid sequence in the M3-M4 extracellular loop. These two versions only differ by 8-11 amino-acids (Sommer et al., 1990; Monyer et al., 1991); yet display profoundly disparate effects on receptor function, in particular on the rate of onset and recovery from desensitisation. The ‘flip’ isoforms of subunits, which predominate before birth and continue to be expressed after birth, desensitise more slowly than the ‘flop’ isoforms, hence prolonging synaptic transmission. ‘Flop’ isoforms however, are low in abundance before postnatal day eight, but are upregulated to approximately the same
levels as ‘flip’ in the adult. Receptor composition, i.e. what percentage of receptors contains ‘flip’ versus ‘flop’ isoforms of subunits, is a key determinant of the sensitivity of receptors to allosteric modulation (Sommer et al., 1990). Hippocampal CA3 pyramidal cells for example express only ‘flip’ subunits, and therefore desensitise more slowly. Other splice variants to be identified in AMPA receptors are the C-terminal splice variants in GluR2 and GluR4. Both subunit C-terminals can be spliced into long and short forms, however the effects of these C-terminal splice variants are still unknown. One suggested role is that they possibly bind to different intracellular proteins, and thus play a role in receptor targeting and trafficking (Gallo et al., 1992; Kohler et al., 1994).

The other form of post-translational modification to which AMPA and kainate, but not NMDA, receptors are subjected, is RNA editing. This is a process in which selected adenosines are deaminated to inosines by dsRNA adenosine deaminases (Reuter et al., 1995), resulting in single amino-acid exchanges (Seeburg et al., 1996). Inosines base pair in a manner identical to guanosines, which results in a change in the amino-acid codon. One key RNA editing site that profoundly affects AMPA receptor function is the ‘Q/R’ editing site in the M2 domain of the GluR2 subunit. In the GluR2 primary transcript at the ‘Q/R’ editing site, a glutamine codon (CAA) can be edited to an arginine (CIG). This arginine GluR2 version causes the receptor in which it is contained to exhibit low Ca$^{2+}$ permeability (Hume et al., 1991), low single channel conductance (Swanson et al., 1996), and the channel shows an approximate linear current-voltage relationship (Verdoon et al., 1991; Hume et al., 1991). GluR2 subunits without this arginine substitution form channels with high Ca$^{2+}$ permeability and a double rectifying current-voltage relationship (Hollman et al., 1991). The vast majority of GluR2 subunits in healthy adult animals (> 99%) contain the edited form of GluR2, resulting in low Ca$^{2+}$ conductance (Bruscet et al., 1995).

Another key editing site is found in an Exon in the M3-M4 loop immediately preceding the ‘flip/flop’ splice site. Editing at this so-called ‘R/G’ site in GluR2, GluR3, and GluR4, but not GluR1 subunits results in the native arginine codon (IGA) being replaced with a glycine codon (AGA). Receptors containing these
edited sites are able to more quickly recover from desensitisation (Lomeli et al., 1994). As with ‘Q/R’ editing the majority (80-90%) of subunits are edited to glycine in the adult animals. Agonist EC$_{50}$, affinity, activation and desensitisation properties all vary with alternate splicing and RNA editing among the various subunits, providing the CNS with a elegant means of fine tuning excitatory synaptic function (Erreger et al., 2004).

Protein phosphorylation also plays a key role in the regulation of neural function. AMPA receptors subunits are targets for phosphorylation, and are directly phosphorylated on at least 12 distinct sites, by PKA (cAMP-dependent protein kinase) (Knapp et al., 1990), CamKII (Ca$^{2+}$-calmodulin-dependent protein kinase II) (McGlade-McCulloh et al., 1993) and PKC (protein kinase C) (Wang et al., 1994a). The major sites on GluR1 include Ser831 and Ser845, which are phosphorylated by PKC/CamKII and PKA respectively. Phosphorylation of these sites leads to a 40% potentiation of the peak amplitude of whole-cell glutamate-gated-currents, and an increase in the open-channel conductance of the receptor (Roche et al., 1996; Mammen et al., 1997; Barria et al., 1997). GluR2 subunits are phosphorylated at Ser863 and Ser880 on its C-terminal domain by PKC (Matsuda et al., 1999; Chung et al., 2000; McDonald et al., 2001). Intriguingly Ser880 is located in a PDZ-consensus domain, which may in turn regulate the interaction of GluR2 with the PDZ, and this has been suggested to be important with regard to the regulation of the synaptic targeting of AMPA receptors and subsequent synaptic plasticity (see section 1.6.2.2). GluR4 is also phosphorylated at Ser830 and Ser842, the function of which is unclear (Carvalho et al., 1999).

1.2.3. Ligand-agonist binding core

The ligand-binding domain of the AMPA receptor subunit occurs as a highly conserved amino-acid binding pocket thought to exist in all glutamate receptors (Oh et al., 1993 & 1994; Sun et al., 1998). This pocket is formed from 2 globular domains, S1 and S2 (see Fig 1.3.), which are found in the sequence adjacent to the M1 domain on the N-terminus and on the M3-M4 extracellular loop respectively. It was initially hypothesised that these two domains formed a bilobular structure in
which the two lobes of the binding pocket were in dynamic equilibrium between the open and closed states, and that when a ligand bound that this stabilised the closed form of this ‘clamshell’ like structure (Sternbach et al., 1994; Kuusinen et al., 1995; Tygesen et al., 1995; Ivanovic et al., 1998; Keinanen et al., 1998). With this precisely defined hypothesised topography, Keinanen’s group in 1995 designed a water soluble mini-receptor composed of only the agonist binding core from the GluR4 subunit (S1 and S2 joined by a peptide linker) (Kuusinen et al., 1995). The isolated domains retained selectivity for AMPA receptor-preferring ligands, which bound with a $K_D$’s similar to those established for full-length receptors. This was followed up in 1998 by Armstrong and colleagues when they fashioned a series of rat S1-S2 GluR2 constructs, which were not only producible in large quantities in a functionally active state, but also enabled precise crystal structural analysis of the agonist-binding cores by X-ray diffraction, confirming the suspected two-domain globular protein structure (Armstrong et al., 1998). It was also discovered that the ligand-binding pocket appeared to be entirely contained within a single subunit rather than at the interface of 2 subunits as in many other receptors. Further to this it was found that the S1-S2 fragment in either the apo or in the ligand-bounded state, crystallised into a dimeric arrangement, with the area of the dimer interface postulated to be around $1500 \text{Å}^2$. These studies (Robert et al., 2001; Sun et al., 2002) also allowed the identification of the exact residues in the protein that form hydrogen bonds with the ligand that occupies the binding site, and also suggested that indirect hydrogen bonding of ligands to residues in S1-S2 via water molecules was important in the mechanism of binding. The residues Pro478, Thr480 Arg481 (all in S1) and Ser654, Thr655, and Glu705 (in S2 domain) make direct hydrogen bonds with glutamate, while the aromatic side-chain of Tyr450 is also thought to form an electron-dense ring structure above the ligand-binding site in GluR2, which is important for agonist potency and desensitisation kinetics. Mutation of Arg481 for example to a lysine results in the loss of all function (Kawamoto et al., 1997). One question unresolved however was how the binding of ligand in S1-S2 facilitated channel opening.
1.2.4. Channel activation

AMPA receptors activated by their native ligand glutamate, mediate the fast components of the excitatory synaptic potential/current. Channel activation, requires at least two molecules of bound agonist (Clements et al., 1998), and induces rapid opening of the channel to multiple conductance levels with a high peak open probability. In reality, up to four molecules of agonist can bind and the average conductance of the channel depends on the number of binding sites occupied by the agonist molecules (Rosenmund et al., 1998). Further investigation into the novel crystallised GluR2 S1-S2 constructs highlighted that they underwent large conformational changes upon binding agonists, switching from a relaxed open-cleft apo conformation to a constrained closed-cleft conformation with a degree of closure in the order of $\sim 21^\circ$. This agonist binding and domain closure is suspected to be a two-step procedure, involving both a ‘docking’ and ‘locking’ action. During docking the agonist binds to residues, the arginine side chain (Arg485) on helix D, in S1 (via its $\alpha$-amine and $\alpha$-carboxyl groups). Following this, the bilobular structure ‘locks’ via the rotation of S2 towards S1 resulting in the closure of the binding cleft. With the agonist partially secured in the binding cleft (S1-bound) the $\gamma$-carboxyl group makes hydrogen bond contacts with the main-chain peptide bond and the hydroxyl group of a conserved threonine side chain in the S2 domain. This electrostatic attraction occurs in all iGluR structures (Mayer, 2006). A further intriguing feature of the glutamate receptor families is how agonist efficacy correlates with the change in closure of the clamshell-like domain. Antagonists for example, cause minimal changes in the degree of domain closure, partial agonists have intermediate effects, and full agonists induce much larger changes (Armstrong et al., 1998; Armstrong et al., 2003; and Erreger et al., 2004). This is best highlighted by a series of 5-substituted willardiines that differ in the size of a halide substituent, for which an increase in substituent size sterically hinders domain closure in a graded fashion (Jin et al., 2003a/b).

This closure of the binding cleft in the S1-S2 dimer was also shown to result in a separation between the portions of the receptor coupled to the ion channel by $\sim 8$ Å. The movement of S2 towards S1 during the transition from the open cleft of the apo
state to the closed cleft of the glutamate-bound complex, produces a scissor-like outward motion of the linkers connecting the ion-channel transmembrane segments to the ligand-binding core (Mayer, 2006). It has therefore been suggested that this direct mechanical coupling of the agonist-induced domain closure to the opening of the ion channel is mediated by the physical separation of the ‘linker’ region of the protein.

1.2.5. Receptor desensitisation

One striking feature of AMPA receptor responses is the rapid onset and extent of desensitisation induced in response to sustained application of agonist (Mayer and Armstrong, 2004; Erreger et al., 2004). This rapidly induced desensitisation is the result of a conformational change intrinsic to the receptor, specifically the breakdown of the dimer interface. Evidence for the role of the stability of the dimer interface as the primary determinant of desensitisation is abundant. Receptor mutagenesis studies that attenuated desensitisation resulted in a more stable dimer complex, while mutations that disrupted the dimer-interface accelerated the onset and extent of desensitisation (Sun et al., 2002). It is proposed that during desensitisation the rearrangement of the dimer interface results in the disengagement of the conformation alterations induced by the closure of the agonist binding cleft in response to the activation of the channel (Sun et al., 2002). Cyclothiazide, a potent inhibitor of receptor desensitisation, stabilises the formation of dimers in the isolated GluR2 binding core, and does so by binding at the base of this interface establishing H-bond contacts with the alternatively spliced serine side-chain of the ‘flip’ splice variant, effectively ‘gluing’ the subunits together. Amino-acid substitutions in the ‘flip/flop’ variants that regulate sensitivity to cyclothiazide lie on the dimer interface of the agonist-binding core (Mayer and Armstrong, 2004). Converting Leu507 in GluR3 to a tyrosine residue abolishes desensitisation in GluR3 containing AMPA receptors (Stern-Bach et al., 1998). It is obvious that both activation and desensitisation (Fig 1.4.) therefore are highly conserved processes that employ a common structural element, namely the dimer interface.
Fig 1.4. Schematic diagram illustrating a model for glutamate receptor activation and desensitisation

The relationships between conformational changes at the ligand-binding core, the dimer interface and the ion channel gate are shown. Two subunits of the tetrameric structure are shown. The domains of the ligand-binding core are labelled D1 and D2 (blue and red), and the transmembrane segments of each subunit are represented by the green cylinders. Each subunit binds a single molecule of agonist (yellow) and can exist in three different conformational states: closed, open and desensitised. Post agonist-binding domain two rotates towards domain 1, and results in channel activation. Closure of domain one towards domain two disrupts the dimer interface and results in desensitisation.
1.3. Kainate Receptors

Kainate receptors display a relatively restricted distribution in the CNS, and are found primarily in the inner laminae of the neocortex and cingulate cortex, caudate putamen, the CA3 of the hippocampus, reticular thalamus and the hypothalamic median eminence (Huettner, 2003). Kainate receptors share ~30-35% sequence identity with AMPA and 10-20% with NMDA receptors, and are thought to adopt the same membrane topology. There are a total of 5 subunits that fall into two families based on sequence homology and agonist binding properties. GluR5/6/7 are 70% identical and are thought to form low affinity receptors, whereas KA1/2, which are also 70% identical but share only 40% sequence homology with the kainate GluR subunits are thought to form heteromeric high affinity channels (Hollmann and Heinemann, 1994). These receptor subunits are capable of forming both function homomeric and heteromeric ligand-gated channels, and similarly to AMPA receptors are subject to extensive mRNA editing. This editing regulates key permeation properties of the receptor (Sommer et al., 1991), with editing at the Q/R site determining single channel conductance and calcium permeability (unedited receptors display a higher calcium permeability and a higher unitary conductance). GluR6 also displays two additional sites of RNA editing in the first transmembrane domain (Kohler et al., 1993). Whilst AMPA and NMDA receptors are predominantly located post-synaptically kainate receptors are also located pre-synaptically at many synapses where they are thought to possibly modulate transmitter release. They are also thought to play a key role in the formation of NMDA receptor-independent LTP, in the mossy fibre pathway in the hippocampus (Harris and Cotman, 1986; Marchal and Mulle, 2004)(see section 1.6.2.).

1.4. NMDA Receptors

Ionotropic NMDA receptors in contrast to AMPA receptors mediate the slow component of excitatory transmission in the CNS (Coan and Collingridge, 1987). These channels are heterogeneously distributed across the CNS with high levels of expression in the hippocampus (CA1), thalamus and the cortex. They display large
single channel conductances (40-50pS) and are highly permeable to Ca\(^{2+}\) in addition to Na\(^{+}\) and K\(^{-}\). Six NMDA subunits exist; NR1, NR2A-D and NR3, and display the lowest sequence identity of all the glutamate receptors (18% for NR1 and NR2). NMDA receptors adopt the same membrane topology as AMPA receptors and form tetrameric structures, composed of heteromers of two NR1 subunits and 2 NR2 subunits (Laube et al., 1998). The two NR2 subunits need not be identical in the assembly, however the kinetic properties of the resultant receptor are highly dependent on subunit composition. For example, deactivation times for receptors are determined by the type of NR2 subunit present: NR2A (50msec) < NR2C (300msec) = NR2B (280msec) << NR2D (1.7s) (Vicini et al., 1998). These ‘modulatory’ NR2 subunits control other key properties such as glycine sensitivity, single channel characteristics and the strength of the Mg\(^{2+}\) block (see below). The calcium conductance of the receptor is controlled by a specific extracellular region (C terminal to M3), unique to the NR1 subunit called DRPEER, which acts as a Ca\(^{2+}\) binding site and causes a constriction of the channel (Watanabe et al., 2002).

A unique feature of the NMDA receptor is that their activation is not solely reliant on the binding of glutamate; a co-agonist, glycine, is also required to bind to elicit channel activation. The NR1 subunit contains the glycine-binding site, whereas NR2 subunits contain the glutamate-binding site. In point of fact, the binding of two molecules of glycine and two of glutamate are required for full channel activation (this also provides further evidence supporting the tetrameric nature of the receptors) (Clements et al., 1998). An additional feature, perhaps the most critical, of the NMDA receptor is that it contains a voltage-dependent channel-binding site for Mg\(^{2+}\). This physical block renders the channels inactive at rest and is only relieved upon depolarisation of the post-synaptic membrane (generally brought about through AMPA receptor activation). This voltage-dependant Mg\(^{2+}\) block, the slow gating of the receptors, and their higher Ca\(^{2+}\) permeability are key properties of the receptors that make them the ideal candidates as activity ‘coincidence detectors’ in the CNS (Section 1.6.2.).
1.5. Roles of Glutamate in the CNS

Glutamate is the major excitatory transmitter in the CNS, and as such, plays a crucial role in regulating various aspect of normal function. Glutamate acting through its varied receptors, outlined previously in the introduction, regulates normal excitatory neurotransmission in the majority of neurones in the CNS, which is imperative for routine synaptic function. It is also critical for in key process known as ‘synaptic plasticity’, the ability of neurones to modify the strength of their connections with one another. However, an imbalance in glutamatergic function (over-excitation) can have detrimental results, resulting in ‘excitotoxicity’, which can lead to both cell damage and death.

1.5.1. Synaptic neurotransmission

Synaptic neurotransmission is a tightly regulated operation intimately involving the ionotropic receptor subtypes, especially the AMPA and NMDA subtypes. Glutamate is released from vesicles (10-100mM concentration) in pre-synaptic terminals following depolarisation of the nerve terminal. It is released in a quantal manner via a Ca$^{2+}$-dependant mechanism, involving N- and P/Q type voltage-dependant Ca$^{2+}$ channels (Birnbaumer et al., 1994). Release is stringently controlled by a host of presynaptic receptors including; Group II and III mGlur’s, cholinergic, adenosine, κ-opioid, GABA-B and cholecystokinin receptors (Nicholls, 1998; Meldrum, 2000); with released glutamate activating both post-synaptic AMPA and NMDA receptors, and most probably pre-synaptic receptors as well. Postsynaptic AMPA receptors mediate the fast-excitatory (depolarisation) component of neurotransmission, while NMDA activation results in slower more prolonged activation. Both receptors are located in the plasma membrane where they are localised to post-synaptic densities. Released glutamate is recycled via active uptake from the synaptic cleft via amino acid transporters found on both neuronal and glial cells. It is essential to remove glutamate, not only to preserve the integrity of synaptic transmission, but to also prevent excitotoxic damage due to elevated levels of glutamate. Five types of Na$^+$-dependant glutamate transporters are expressed within the mammalian CNS: GLT, GLAST, EEAC1, EEAC3, EEAC5 (Lehre and Danbolt, 1998; Seal and Amara,
GLT and GLAST are expressed predominantly in glial cells, while EEAC1, 3, 5 are restricted to neuronal expression. Glutamate transporters are highly expressed in the post-synaptic membranes, especially in neurones, and in some cases are 15 times more abundant than the AMPA receptors. These transporters utilise the electrochemical gradients of $\text{Na}^+$, $\text{K}^+$ and $\text{H}^+$ to facilitate the uptake of glutamate. It is worthy to note that glutamate can also be released by the reverse operation of glutamate transporters (Meldrum, 2000). This occurs when the $\text{Na}^+$ and $\text{K}^+$ gradient across the membrane is reduced during instances of cerebral ischaemia for example (Levy et al., 1998). In total, two thirds of total brain energy metabolism is related to the reuptake and recycling of glutamate (Shulman et al., 2002), and the process of excitatory synaptic neurotransmission, which it facilitates, is fundamental to normal functionality.

1.5.2. Synaptic Plasticity

Synaptic plasticity in the most basic sense is the ability of the connection, or synapse, between two neurones to change in strength. A more ‘philosophical’ view is that it is the ability of neurones to not only effectively convey information to one another, but to alter the efficiency with which they do it. This phenomena can be modified in both directions, resulting in either an increase in synaptic strength (a potentiated synapse), or a decrease (a depressed synapse). The flexibility of the system, allowing it to be potentiated, depressed and modified inbetween makes it possible to encode vast amount of information in this neural model. The strength of a synapse is also crucially reliant on the number of ion channels and receptors it has (Debanne et al., 2003). AMPA along with NMDA receptors, through their role in neurotransmission as outlined above, are also crucial in the manifestation, maintenance and modification of synaptic plasticity.

1.5.2.1. Long-term-potentiation (LTP)

LTP first observed in the rabbit hippocampus by Terje Lømo in 1966 is the most studied form of synaptic plasticity, and is entirely reliant upon the activation of glutamatergic receptors. Lømo observed that a single pulse of electrical stimulation to the perforant pathway elicited an excitatory post-synaptic potential (EPSP) in the
dentate gyrus of the hippocampus. When a high-frequency train of stimulation was imparted, it produced larger prolonged EPSPs compared to responses elicited by a single stimulus: hence LTP (Fig 1.5.) (Lømo, 1966). The majority of researchers regard LTP and its opposing process, long-term-depression (LTD), as the cellular basis of learning and memory. LTP is however not a unitary phenomena, but varies depending on which synapse it is being studied, at what time point in development, and how it is triggered (Malenka and Bear, 2004).

![Graphical representation of various forms of synaptic plasticity](image)

**Fig 1.5. A graphical representation of various forms of synaptic plasticity**

Long-term-potentiation (LTP) is induced by tetanic stimulation, typically 100 Hz for 1 sec. This stimulation paradigm results in an increase in the amplitude of the fEPSP response which settles into a new, more elevated level. A weak tetanic stimulus results in a an initial potentiation which decays back to baseline levels after ~20-30 min: a short-term-potentiation (STP). Repetive low frequency stimulation, 1 Hz for 10 min, results in a decrease in the amplitude of the fEPSP response, which settles at a lower level, known as long-term-depression (LTD).

LTP as intimated above can be studied at multiple synapses from varied regions of the brain, including the cortex and thalamus. However, it is most frequently studied in the hippocampus, a limbic structure located in the medial aspect of the temporal lobe, which is implicated as playing a major role in memory storage and the emotional aspects of behaviour. This is because, when experimentally removed and sliced the hippocampus provides an ideal model with which to record neuronal
activity and investigate synaptic plasticity, due to its 1) highly defined laminated structure (Amaral and Witter, 1989), allowing easy and accurate discrimination of cell types and subsequent electrode placement, and 2) its excellent mechanical stability, allowing prolonged and stable recordings to be attained.

**Fig 1.6. The laminated structure of the hippocampus**

Input from the perforant pathway (lateral, LPP, and medial, MPP, pathways) synapse with the dentate gyrus (DG) granule cells. Mossy fibres (MF) project from the DG to the CA3 pyramidal cells. Schaffer collateral fibres (SC) run from the CA3 and synapse with CA1 pyramidal cells. Commissural fibres (AC) from the opposite hemisphere’s CA3 also synapse with the CA1. The major output of the hippocampus is from the CA1 to the entorhinal cortex. Recordings are traditionally obtained from the CA1 stratum radiatum (right-hand electrode) in response to stimulation of the SC/AC fibres (left-hand electrode). An example field excitatory post-synaptic potential (fEPSP) is also shown.

Structurally (Fig 1.6.), the hippocampus receives its major input from the entorhinal cortex, via the perforant pathway. Axons arising primarily from layers II and III but also IV and V of the entorhinal cortex project to the granule cells of the dentate gyrus and the pyramidal cells of the CA3 hippocampal region (from layers II/IV), and to the CA1 and subiculum (from layers III/V). The DG granule cells give rise to the mossy fibres that synapse with the pyramidal cells of the CA3 and CA2 regions of the hippocampus. Multiple granule cells are capable of synapsing onto a single CA3 pyramidal cell (LTP here is NMDA receptor independent, and is thought to involve pre-synaptic kainate receptors). The CA3 pyramidal cells project excitatory collaterals, the Schaffer collaterals, to synapse on the pyramidal cells in the CA1 region. The hippocampal commissurals are fibres arising from the contralateral hemisphere’s CA3 regions that synapse on the CA1 of the opposite side (cross
hemispheres) (LTP here is NMDA receptor-dependant). The principle output pathways of the hippocampus are the perforant path, the cingulum bundle, and the fimbria/fornix, which all arise from field CA1 and the subiculum.

LTP in the hippocampus is investigated most commonly in the CA3-CA1 Schaffer collateral/commissural pathway, as outlined above. Here, repetitive stimulation results in the coordination of neuronal activity in the Schaffer collateral axons, which in turn leads to pre-synaptic neurotransmitter release in a Ca$^{2+}$-dependant manner. Released glutamate activates postsynaptic AMPA receptors, resulting in the initial depolarisation of the postsynaptic cell. The profound voltage-dependent Mg$^{2+}$ block of the NMDA receptor-channel is subsequently relieved following this strong post-synaptic depolarisation, allowing Ca$^{2+}$ (and Na$^+$) to flood into the post-synaptic cell through the active NMDA receptor. This rise in intracellular Ca$^{2+}$ is the critical trigger for LTP formation.

Calcium is a crucial mediator of this phenomenon, as manipulation of its levels in the postsynaptic cell influences the form of synaptic plasticity induced. Increases in calcium that are NMDA-dependant but do not reach the ‘threshold’ needed to induce LTP can generate a short-term potentiation (STP), which decays to baseline levels within $\sim$20-30 min. LTD is also calcium-dependant and results in a long lasting decrease in synaptic strength, but is brought about by an alternate distinct stimulation paradigm (several minutes of low freq stimulation).

The post-synaptic (intracellular) mechanisms underlying LTP induction are not yet fully determined. What is clear, is that NMDA receptors form large post-synaptic complexes of $>600$ proteins, known as post-synaptic-densities (PSD’s). These complexes work as a ‘hub’, in order to transduce receptor activation into downstream signals, which induce and maintain the expression of LTP. However it is still unclear exactly which molecules are mediators and which are modulators of the process (reviewed in Malenka and Bear, 2004). Indeed it seems that there may be multiple intracellular cascades that are capable of inducing LTP, but whatever the mode of induction certain proteins are required no matter how it is induced. It is...
clear that calcium/calmodulin-dependant protein kinase II (CaMKII) is a definite mediator of LTP (Lisman \textit{et al.}, 2002), with other molecules such as PKA, PKC, PI3 kinase, the tyrosine kinase Src, and MAPKs also playing key roles in the formation of LTP (Malinow \textit{et al.}, 1989; Sweatt, 2004; Thomas and Huganir, 2004; Man \textit{et al.}, 2003; Kalia \textit{et al.}, 2004). These signalling molecules in turn activate key transcription factors such as CREB, in addition to immediate early genes such as zif268 (Silva \textit{et al.}, 1998; Lynch, 2004). This in turn, is hypothesised to result in the generation of ‘synaptic tags’ (plasticity related proteins), which help to stabilise the initial increase in synaptic strength (Frey and Morris, 1998).

The phenomenon of LTP is clinically relevant, as it is believed by many to be the major neural substrate underlying learning and memory (cognition) (Lisman, 2000). Many clinical conditions including depression, schizophrenia, Alzheimer’s and Parkinson’s disease all display cognitive deficiencies, and for that reason compounds which have the ability to augment LTP may have potential therapeutic relevance in the afore mentioned conditions.

1.5.2.2. The role of AMPA receptor regulation in LTP

One of the most controversial debates surrounding LTP over the last decade or so was if the increases witnessed in synaptic strength were mediated primarily by a pre- or post-synaptic mechanism. The now generally held belief is that the change in strength witnessed during LTP is mediated by a post-synaptic modification of AMPA receptors. Indeed, during LTP the AMPA-mediated portion of the EPSC is increased to a greater extent than the NMDA component.

The most obvious way of ‘increasing synaptic strength’ post-synaptically is via the insertion of extra receptors. In support of this, AMPA receptors have been shown to be rapidly delivered to active synaptic membranes due to repetitive NMDA receptor activation (Shi \textit{et al.}, 1999; Song and Huganir, 2002), via activity-dependant changes in AMPA receptor trafficking. Many neurones also have so called ‘silent’ synapses that have functional but quiescent NMDA receptors (due to the Mg$^{2+}$ block), but lack any AMPA receptors (Gomperts \textit{et al.}, 1998; Gasparini \textit{et al.}, 2000; Isaac, 2003).
AMPA receptors are recruited, trafficked, and inserted into these synapses during LTP, and as such, this process may mediate a major component of the potentiation of synaptic transmission during LTP. Another possible way of increasing synaptic strength is via direct modification of the biophysical properties of AMPA receptors themselves. Protein phosphorylation of AMPA at Ser 831/835 (especially on GluR1 subunits) by PKC and CaMKII has been shown to result in increases in single channel conductance (Benke et al., 1998; Lee et al., 2003). Conversely, the cellular effects of LTD are associated with a reduction in AMPA receptor numbers at the synapse, and also de-phosphorylation of key Ser residues on GluR1. This however is not to say that presynaptic mechanisms don’t contribute at all to the increased synaptic fidelity witnessed with LTP. The roles of retrograde messengers as presynaptic modulators are still being investigated, with attention now focusing on molecules such as brain derived neurotrophic factor (BDNF) and cell adhesion molecules as potential mediators (Poo et al., 2001).

As the extent of depolarisation during induction is believed to be the most critical factor for the activation of NMDA receptors and hence the overall magnitude of LTP induced (Xia and Arai, 2005), modification of AMPA receptor function, either directly via changing channel properties, or indirectly by increasing the number of functional receptors present, seems to be the simplest way of increasing post-synaptic depolarisation, ensuring efficient and robust LTP. Work in this thesis, using the hippocampal model outlined above, will attempt to investigate what effects modifying AMPA receptor kinetics, through the use of novel ligands targeting the AMPA receptor, have on baseline synaptic activity and in an established and characterised LTP model; and whether or not these potential effects could in some way be of benefit in relevant disease states.

1.5.3. Excitotoxicity
Glutamate in addition to its positive effects in mediating excitatory neurotransmission has an altogether more detrimental role in the CNS. Excessive activation of glutamatergic receptors can also evoke neuronal dysfunction, cellular damage and death, a phenomenon that is termed ‘excitotoxicity’. Lucas and
Newhouse first described this process in 1957, when they injected L-glutamate into murine eyes, and were surprised to find that it resulted in the destruction of the inner layers of the retina. These findings were replicated, confirmed, and built upon by Olney who illustrated that kainate produced brain lesions in immature animals lacking a fully developed blood-brain-barrier (Olney, 1969). In addition to this, experiments with NMDA receptor antagonists such as MK-801 demonstrated that by blocking glutamatergic receptors, these compounds could be neuroprotective in vivo (Simon et al., 1984; Ozyrut et al., 1988). Further evidence has shown that high-concentrations of glutamate causes over-activation of glutamate receptors that results in both ionic disturbances in cells (neurones) and the activation of Ca$^{2+}$-mediated processes, as outlined above. Activation of AMPA and NMDA receptors by glutamate results in changes in intracellular concentrations of Na$^+$ (Cl$^-$) and Ca$^{2+}$, and the ensuing excitotoxicity can be separated into two distinct phases. The first is an acute phase that is marked by immediate toxic cell swelling due to rapid Na$^+$ (and Cl$^-$) influx through activated glutamate receptors (AMPA) (Rothman, 1985). However, removal of extracellular Na$^+$ even though it eliminates cell swelling, does not prevent cell death (Olney et al., 1986). Cells undergo a second phase of delayed neurodegeneration, which is entirely Ca$^{2+}$ dependant.

The regulation of Ca$^{2+}$ levels within neurones is essential to maintaining normal functionality, as it plays key roles in diverse processes ranging from regulating intracellular signalling cascades, growth and differentiation, synaptic activity (exocytosis) to governing membrane excitability. With this in mind, neurones posses specialised homeostatic mechanisms to maintain tight control over cytosolic calcium levels via control of influx, efflux, buffering and internal storage. Schlaepfer and Bunge in 1973 were the first to highlight the ‘negative’ role of calcium, specifically that degeneration of isolated axons intimately involved extracellular Ca$^{2+}$ ions. It was further elucidated that excessive Ca$^{2+}$ influx or release from intracellular stores essentially overloads the capacity of the inherent Ca$^{2+}$-regulating mechanisms resulting in inappropriate activation of Ca$^{2+}$-dependant process that are normally dormant or running at low levels. Over-activation of processes involving proteases, lipases, phosphatases, and endonucleases (metabolic derangement) leads to cell death.
structural damage and the formation of oxidative free radicals, such as NO$, which eventually lead to cell death (Kristian and Siesjo, 1997). Ca$^{2+}$ can therefore in addition to regulating key cellular processes, be considered a key mediator of cell death. In glutamate induced excitotoxicity the increased cellular calcium levels are a result of increased influx through both AMPA (GluR2 absent) and NMDA receptors, activation of voltage-sensitive Ca$^{2+}$ channels as a result of depolarisation primarily by AMPA, and release of Ca$^{2+}$ from intracellular stores (Choi, 1988). This dysregulation of Ca$^{2+}$ homeostasis leads to activation of distinct intracellular signalling cascades, described above, that eventually results in cell death. Excitotoxicity is clinically relevant and a cause of cell death in both acute insults such as stroke, hypoxic-ischaemia and trauma, and in chronic neurodegenerative diseases such as Alzheimer’s, Parkinson’s and Huntington’s disease. For example, the main phase of the pathogenetic process leading to schizophrenia is the loss of synaptic connectivity below critical level, which has been proposed to be partly due to excitotoxicity (specifically in oligodendroglia) (Yao & Reddy, 2005).

1.6. Pharmacological Compounds Targeting the AMPA Receptor

It is now strikingly obvious that both the AMPA and NMDA ionotropic glutamatergic receptors play key roles in neurotransmission, synaptic plasticity and even have damaging effects mediated by their ability to induce ‘excitotoxicity’. Historically, research into potential clinical targets involving the glutamatergic system has focused primarily on the key role of the NMDA receptor in neurotransmission. Studies in the early 1980s demonstrated that NMDA receptor antagonists, such as 2-amino-7-phosphonoheptanoic acid, blocked seizures in rodent models of epilepsy (Croucher et al., 1982), but more importantly had dramatic neuroprotective effects against ischaemic brain injury (stroke) (Simon et al., 1984). However, the positive results witnessed in animal models have so far been unsuccessfully replicated in clinical trials in patients with ischaemic stroke. Although NMDA research is still ardently underway, attention has begun to focus on the AMPA receptor as a more viable pharmacological target, for subtle modulation
of receptor and therefore cerebral function. As a result of this, several categories of AMPA receptor ligands have been developed; among them are agonists, antagonist and modulators.

1.6.1. AMPA receptor agonists

There is now an abundance of AMPA receptor agonists, and many as is the case with AMPA itself, have been derived from classic structure activity studies utilising ibotenic acid, quisqualic acid and willardiine. Glutamate, quisqualate, AMPA and ACPA are full agonists, whilst kainate and the 5-substituted willardiine, (S)-5-flourowillardiine, act as partial agonists at the receptor (Fig 1.7.A). Competition studies utilising $[^3]$HAMP A binding to GluR1/2/3 illustrate a rank potency of quisqualate > AMPA = domate > glutamate > kainate for agonists. An interesting feature of AMPA agonists is that they vary dramatically in the amount of receptor desensitisation they induce. Glutamate and AMPA induce rapidly desensitising responses, whereas the partial agonist kainate shows little desensitisation response. Indeed the degree of desensitisation correlates precisely with the degree of dimerisation induced between the pairs of subunits within the receptor (reviewed Stensbol et al., 2002). AMPA agonists have little clinical value themselves, yet are invaluable tools for helping further elucidate AMPA receptor structure and function, as well as helping characterise novel antagonists and modulators.

1.6.2. Competitive AMPA receptor antagonists

Competitive antagonists are compounds that compete with the native neurotransmitter for the same ligand-binding site, effectively blocking agonist binding by trapping the ligand-binding domain in a wide-open conformation (Fig 1.7.B). The first potentially clinically useful competitive AMPA receptor antagonists, the quinoxalinediones, were introduced in 1988 (Drejer and Honore, 1988). CNQX and DNQX whilst effective competitive antagonists of AMPA receptors also displayed activity at the glycine-binding site of the NMDA receptor (Birch et al., 1988), and were therefore deemed not selective enough. By modifying
Fig 1.7. Chemical structures of key ligands for the glutamatergic AMPA receptor

Ligands include examples of: agonists (A), competitive antagonists (B) and non-competitive antagonists (negative allosteric modulators) (C).
these initial structures, more potent and selective antagonists were obtained, including NBQX, PNQX and YM872. NBQX showed robust neuroprotection, reducing excitotoxic cell death (Sheadown et al., 1990; Gill et al., 1992) and anticonvulsant activity (Chapman et al., 1991) in a variety of disease related models. Even though it was more selective, it displayed poor water solubility and failed clinical trials due to its profound nephrotoxicity (Kohara et al., 1998).

More recently competitive antagonists with improved potency, higher specificity, increased water solubility, longer duration of action in vivo, and reduced nephrotoxicity have been developed. Based again around the original quinoxalinediones structure, compounds such ZK200775 (Turski et al., 1998), and decahydroisoquinolines such as LY293558 (O’Neill et al., 1998), are highly active in in vivo models and in the case of ZK200775 have entered clinical trials for the treatment of stroke. SPD502 (NS1209), an isatine oxime modification of the classical quinoxalinediones structure, is a further example of a competitive AMPA receptor antagonist that is neuroprotective in vivo (Neilsen et al., 1999; McCracken et al., 2002).

1.6.3. Non-competitive AMPA receptor antagonists

A non-competitive antagonist (negative allosteric modulator) is a compound that blocks receptor function by binding to sites distinct from the agonist recognition site (Fig 1.7.C). The most extensively studied of these compounds are the 2,3-benzodiazepines, typified by GYKI 52466 (Solyom and Tarnawa, 2002) and GYKI 53655 (LY300164). These compounds are systemically available, and selective for AMPA over kainate/NMDA receptors, inhibiting AMPA receptor mediated responses in a variety of cell types with low micromolar IC₅₀ potencies. Use of the 2,3-benzodiazepines has enabled precise pharmacological separation of AMPA and kainate receptor mediated events (Rogawski, 1993). Quinazolinone derivatives such as CP-526,427 have also been shown to be non-competitive AMPA receptor antagonists (Chenard et al., 2000), with a binding site distinct from glutamate but overlapping with that of the 2,3-benzodiazepines. YM928 and CP-465,022 are further examples of non-competitive AMPA receptor antagonists (Ohno et al., 2003; Menitti et al., 2003).
1.6.4. AMPA receptor potentiators

The final group, and most important with regard to this thesis, of AMPA receptor ligands to be discussed here are the AMPA receptor potentiators. AMPA receptor potentiators are the common name given to a group of drugs that positively allosterically modulate receptor function. They were developed in the early 1990s, and were found to functionally facilitate AMPA receptor kinetics and thus fast excitatory transmission. This in turn led to the unexpected observation that fast excitatory transmission in behaving animals can be enhanced without causing seizures or excitotoxic damage (Lynch, 2006), and the subsequent inference that enhancement of excitatory function could possibly have beneficial therapeutic effects. With this in mind, these allosteric AMPA receptor modulators were developed further and have been progressively and extensively investigated over the following 15 years.

These compounds function by binding to sites distinct from the agonist recognition site, facilitating receptor function in the presence of the native agonist (glutamate). These modulators have no direct agonist action themselves, but potentiate function by increasing current flux through the activated receptor either by: 1) preventing receptor desensitisation, a process by which the receptor ion channel closes although glutamate remains tightly bound (a long-lasting agonist-bound non-conducting state); 2) preventing receptor deactivation, a process by which the ion-conducting pore of the receptor closes allowing agonist to dissociate from the ligand binding ‘clamshell’; 3) a combination of both the afore mentioned mechanisms (Fig 1.8.) (Arai et al., 1996b & 2002).

It should also be noted here that determining the mode of action of AMPA receptor potentiators, i.e. whether they preferentially effect desensitisation or deactivation, is most clearly demonstrated experimentally by electrophysiological means. Desensitisation is demonstrated by recording the rapidly diminishing AMPA mediated currents from voltage-clamped neurones during sustained application (500ms – 1sec) of AMPA agonists (effects on steady-state current). Deactivation kinetics are demonstrated by recording again from voltage-clamped neurones, but
this time during very rapid application (1ms) of AMPA agonists (effects on rapidly decaying short-lived responses).

To date, through extensive research much has been learnt about the compounds binding sites, biophysical actions and influence on cortical networks. However, there is still an absence of information regarding how these compounds alter complex functional activity in vivo, i.e. their overall effect on cerebral activation. In this regard, the primary aim of this thesis is to investigate the actions of two novel next generation AMPA receptor potentiators, Org 26576 and Org 24448, and attempt to determine, and if possible, explain their effects in vivo. Prior to this however, it is essential to examine and understand the evolution of these AMPA receptor potentiators from their inception in the early 1990s. With this in mind, the several families of structurally distinct AMPA receptor modulators in existence are outlined in detail below.

1.6.4.1. Plant lectins
The plant lectin concanavalin-A was the first compound identified to exhibit a positive modulatory action on AMPA receptors. The action of lectins on excitatory amino-acid responses had previously been studied extensively at the neuromuscular junction of crayfish, locusts and stingrays, and had been shown to rapidly reduce GluR desensitisation (Stettmeier et al., 1983; Evans and Usherwood, 1985; O’Dell and Christensen, 1989). Mayer and colleagues presented the first evidence for a positive effect at mammalian synapses in 1989. They found that concanavalin-A potentiated the peak response to quisqualate (16% increase in peak current), without altering overall channel conductance levels; and also maintained the steady-state current evoked by 100μM quisqualate by 13-times over control levels (profound inhibition of desensitisation) (Mayer et al., 1989). A further study confirmed that the augmentation of the steady state current was not due to a change in channel conductance but was a result of an increase in the mean channel burst length of the AMPA receptors (Thio et al., 1992).
Fig 1.8. Simplified kinetic scheme for the AMPA receptor and where AMPA receptor potentiators act

Sites and mechanism of action for Ampakine compounds are indicated: R = receptor, A = agonist, RdA = desensitised state of the receptor with the agonist bound Rd = desensitised state of the receptor (adapted from Yamada, 1998, and Black et al., 2000).

- **Ampakines** acts here: Slow both desensitisation and deactivation to varying degrees depending on compound and flip/flop composition of the receptors
- **Aniracetam** acts here: Slows deactivation (channel closing)
- **Cyclothiazide** acts here: Blocks desensitisation / accelerates re-sensitisation and stabilises the agonist bound closure state
1.6.4.2. Pyrrolidinones

The interest in lectins as potential AMPA receptor modulators was superseded by the discovery that the ‘nootropic’ (Greek: ‘toward the mind’) pyrrolidinones, such as piracetam and aniracetam, more therapeutically relevant compounds, were also probable AMPA receptor potentiators. Prior to interest in the pyrrolidinones as AMPA receptor modulators, the compounds had been investigated both in animal models and in clinical scenarios as cerebral homeostatic normalisers, neuroprotectants, cerebral metabolic enhancers, brain integrative agents, and cognitive enhancers. Aniracetam for example reversed the memory impairment in rats induced by clonidine, and also attenuated and reversed amnesia in mice and rats via various modes of induction (scopolamine, cyclohexamide, ECT) (Lazarova-Bakarova and Genkova-Papasova, 1989; Martin et al., 1995). In humans the compounds had been shown to improve learning and memory in both aged and normal healthy individuals (slow cognitive decline), and importantly they also displayed very low toxicity (Lee and Benfield, 1994). Considered by many as the first seminal study into AMPA receptor potentiators, Ito and colleagues in 1990 examined the actions of aniracetam (N-anisoyl-2-pyrrolidinone or RO135057), in both *Xenopus* oocytes expressing recombinant AMPA receptors, and on native AMPA receptors in acute rat hippocampal slices. They found that aniracetam increased AMPA receptor mediated currents in oocytes without affecting GABA/Kainate/NMDA receptors, and increased the size of fEPSP’s in slices with no overt effects on the resting membrane properties. Further work by Isaacson and Nicoll (1991) confirmed that aniracetam primarily functioned by producing profound effects on deactivation by selectively prolonging the time course of fast synaptic currents. These effects were rapid in onset and easily washed out to baseline levels. It should also be noted that aniracetam is ‘flop’ isoform selective (Xiao et al., 1991). However, due to the fact that these compounds contain an -imide group they display very poor blood brain barrier penetration and require large doses to elicit therapeutic effects (low potency) when administered peripherally. The compounds are also very rapidly metabolised (Guenzi and Zanett, 1990).
1.6.4.3. **Thiazides**

A second ‘group’ of AMPA receptor positive modulators chemically related to sulphonamides, was also identified in the early 1990s. Diazoxide, the prototypic thiazide compound (a diuretic), was discovered whilst studying its known action of activating ATP-sensitive potassium channels. Diazoxide reversibly increased the peak amplitude of EPSCs, prolonged the EPSC decay time constant via a direct AMPA receptor mechanism, and was subsequently shown to be an inhibitor of both desensitisation and deactivation of AMPA responses. It was also far more potent than previously identified AMPA receptor potentiatiors (aniracetam) (Tang et al., 1991; Isaacson and Nicoll, 1991; Yamada and Rothman, 1992). Cyclothiazide, a second-generation compound, is by far the most studied of all the AMPA receptor modulators and its characterisation has helped to further understand the precise mechanism of AMPA receptor desensitisation (Fig 1.9.A). Cyclothiazide produces a near complete suppression of desensitisation, but has minimal effects on deactivation kinetics (Yamada and Tang, 1993; Yamada, 2000). Investigation by Partin and colleagues identified that cyclothiazide completely eliminated desensitisation of ‘flip’ AMPA receptor isoforms, but only slowed the entry of ‘flop’ isoforms into the desensitised state (Partin et al., 1994; Yamada, 2000). Further studies utilising site-directed mutagenesis established that a single serine residue, Ser750, in ‘flip’ isoforms is necessary and sufficient to account for cyclothiazide’s potent block of desensitisation (Partin et al., 1995). Cyclothiazide, although a potent blocker of desensitisation has turned out to be best suited as a pharmacological tool, as it lacks good blood-brain-barrier penetration and as such cannot be investigated in in vivo models. IDRA-21, another thiazide derivative, on the other hand displays good blood-brain-barrier penetration and has been shown to potentiate AMPA transmission in the hippocampus in vivo (Bertolino et al., 1993; Uzunov et al., 1995).
Fig 1.9. Chemical structures of positive allosteric modulators of glutamatergic AMPA receptors
Positive allosteric modulators include examples of: thiazides (A), the ‘Ampakine’ family (B), the biarylpropylsulfonamides (C), and the novel Org Ampakines Org 24448 and Org 26576 (D).
1.6.4.4. **Biarylpropylsulfonamides**

Lilly Neuroscience introduced the newest class of AMPA receptor potentiators in 2000. The biarylpropylsulfonamides (Ornstein *et al.*, 2000), of which LY392098 and LY404187 are representative examples, are highly selective, very potent potentiators of AMPA receptor mediated responses (Fig 1.9.C). Both compounds show no selectivity at kainate, NMDA, and voltage-gated ion channels. They display good blood-brain-barrier penetration (Vandergiff *et al.*, 2001), have an extracellular site of action, and increase the potency of the agonist for the receptor ~7-fold (Baumbarger *et al.*, 2001a/b). EC\textsubscript{50} values for both compounds are in the range of 0-2\,µM for GluR1-4 subunits, and LY392098 applied for example at 1.7\,µM in PFC neurones increases AMPA receptor currents 31-fold relative to AMPA alone (Baumbarger *et al.*, 2001a). Threshold concentrations for observable effects are in the range of 10-30nM, making these compounds ~1000 times more potent than cyclothiazide and the Ampakine compounds, and as such are termed ‘high impact’ compounds by the industry. The compounds also display differential selectivity, with LY392098 most potent on GluR4 whilst LY404187 is most potent on GluR2 (Miu *et al.*, 2001). Functionally the biarylpropylsulfonamides have been shown to potentiate responses in pre-frontal cortex neurones both *in vitro* and *in vivo* (Baumbarger *et al.*, 2001), and also *in vivo* they increase the probability of evoked action potential discharge in the hippocampus in response to stimulation of glutamate afferents from the ventral subiculum (Kimball *et al.*, 2000).

1.6.4.5. **Non-specific AMPA receptor potentiators**

PEPA, 4-[2-(phenylsulphonylamino)ethyl-thio]-2,6-difluorophenoxyacetamide, is a sulphonamino compound that also functions as a positive allosteric modulator of the AMPA receptor (Fig 1.9.A). The compound, effective at micromolar concentrations (EC\textsubscript{50} 50\,µM), has no effects on the deactivation of glutamate induced AMPA receptor responses, but potently attenuates the extent of receptor desensitisation, displaying a preference for ‘flop’ spice variant subunits, in sharp contrast to cyclothiazide, and displays subunit specificity such that is effects are greater on GluR3>GluR4>GluR1 subunits (Sekiguchi *et al.*, 1997 & 1998). S18986, (S)-2,3-dihydro-[3,4]cyclopentano-1,2,4-benzothiadiazine-1,1-dioxide, is another compound
that increases AMPA receptor currents in *Xenopus* oocytes (EC\(_{50}\) 35μM), and potentiates fEPSPs in acute rat hippocampal slices (Desos *et al*., 1996; Lebrun *et al*., 2000).

### 1.6.4.6. Benzoylpiperidines / Benzoylpyrrolidines

Synthesised and developed by Gary Lynch at the University of California/Cortex Pharmaceuticals, this group, termed the ‘**Ampakines**’, is by far the most common and widely used of all the AMPA receptor potentiators, and is typified by the prototypic compound CX516 (Fig 1.9.B). Initially structurally derived from aniracetam, compounds such as 1-(1,3-benzodioxol-5-ycarbonyl)-piperidine (BDP) and CX516 (BDP-12) display enhanced potency and efficacy, good brain penetration and reduced metabolic inactivation (Staubli *et al*., 1994b). BDP and CX516 when tested in hippocampal slices elicited fEPSP amplitude EC\(_{50}\) values of 1.5 and 0.3mM respectively, ~100 times more potent than previously reported with other compounds (pyrrolidinones and thiazides) (Yamada, 2000). CX516 also displays moderate ‘flip’ subunit selectivity. *In vivo* however, CX516 still exhibits a relatively short half-life (~1hr) with blood concentrations peaking around 45min after oral administration (Lynch *et al*., 1998), and still relatively low potency. Subsequent further structural modifications including conformational restriction of the amide with rigidification of two rotatable bonds resulted in compounds such as CX554 (BDP-20) and CX614, which are a further ten-times more potent (low micromolar) in behavioural tasks compared to CX516 (Grove *et al*., 2000). Ampakines have been subjected to the most intense *in vivo* and *in vitro* evaluation in both rodents and humans, and as a consequence are arguably the most systematically developed of any all the AMPA receptor modulating compounds. Functionally, the compounds consistently potentiate current flow through the active AMPA receptor; enhance synaptic transmission (Aria *et al*., 1994 &1996a/b) and neural activity (Staubli *et al*., 1994a/b; Hampson *et al*., 1998); modify animal behaviour (Granger *et al*., 1993; Larson *et al*., 1995 & 1996), and augment gene expression (Holst *et al*., 1998). There is however functional diversity within the group concerning their mode of action and potency. CX554 for example has more pronounced effects on receptor desensitisation compared to CX516 (Arai *et al*., 1996a/b), whereas CX614 is equally effective in
influencing both aspects of receptor kinetics (Arai et al., 2000). CX546 on the other hand while structurally similar to CX614 massively prolongs response duration but minimally effects desensitisation.

1.6.4.7. The Ampakines Org 26576 and Org 24448
As mentioned previously, the Ampakines that will be investigated in this thesis, Org 26576 and Org 24448 (Fig 1.9.D), are structurally distinct compounds derived from the first generation Ampakine CX516 (Staubli et al., 1994a). These compounds display a 10-30 fold greater potency when compared to CX516 in potentiating AMPA-mediated electrophysiological responses, with an EC\textsubscript{50} of 8-16 μM in rat hippocampal primary cultured neurons, and both Org 26576 and Org24448 demonstrate selectivity for AMPA receptors when tested at 10 μM against >60 molecular targets including G-Protein Coupled Receptors, ion channels and kinases (NovaScreen Biosciences Corporation, Maryland, USA). Org 26576 and Org 24448 also display antidepressant actions at 3 & 10 mg/kg respectively in the forced swim test, and reduced amphetamine induced locomotor activity at doses as low as 0.1 and 0.3 mg/kg respectively. In addition to this, Org 24448 also shows minimum effective doses of 0.1, 0.1 mg/kg in an \textit{in vivo} screen for anti-psychotic activity and in models for cognition (delayed matching to position (DMTP) task) respectively, compared to 10, 15 mg/kg for CX516 (Cortex Pharmaceuticals, Inc Patent: WO9835950, 1998).

1.7. The Clinical Utility of AMPA Receptor Potentiators
To reiterate, >60% of all neurones in the brain, including all cortical pyramidal neurones and thalamic relay neurones utilise glutamate as their primary neurotransmitter (Javitt, 2004). As glutamatergic neurotransmission is mediated primarily through the ionotropitic glutamate receptors, of which the AMPA receptors mediate the fast excitatory component, it stands to good reason that these receptors are under intense scrutiny as a potential drug development target, a fact highlighted by the number of ligands now available for the AMPA receptor (see above). In support of this I alluded earlier to the concept that the modulation of fast excitatory
transmission could lead possibly to beneficial therapeutic effects, instead of the
excitotoxic damage and seizure activity commonly associated over-stimulation of
glutamatergic receptors. This reasoning has been compounded in recent years by the
emergence of a large body of evidence implicating glutamate neurotransmission, or
lack thereof, in the pathophysiology of various neurodegenerative diseases and
conditions. Deficiencies in glutamatergic neurotransmission are thought to in part
contribute to cognitive impairment/deficits, and there is also growing evidence
suggesting reduced neocortical glutamatergic function and disrupted circuitry
involving glutamatergic signalling may play a direct role in the pathophysiology of
conditions such as cognitive dearth, schizophrenia (psychoses) and depression
(Tamminga, 1999). AMPA receptor potentiators may be therefore of potential
clinical value in conditions such as these, and in point of fact there is a large body of
preliminary evidence supporting this postulation (Li et al., 2001; Marenco et al.,
2002; Coyle et al., 2002).

Ampakines specifically have been shown to be able to facilitate LTP both in vivo and
in vitro (Bertolino et al., 1993; Staubli et al., 1994 a/b; Arai et al., 1996), and
improve both rodent and human performance in behavioural models of
cognition/learning and memory (Staubli et al., 1994; Larson et al., 1995; Zivkovic et
al., 1995; Hampson et al., 1998; Ingvar et al., 1997; Thompson et al., 1995; Lebrun
et al., 2000). AMPA modulators also display significant neuroprotective effects in
NMDA agonist (ibotenate) lesions in the mouse brain (Dicou et al., 2003), and
provided functional, neurochemical and histological protection against 6-
hydroxydopamine lesions in the substantia nigra of rats (O’Neill et al., 2004). They
also display an antidepressant-like activity profile in animal behavioural models
predictive of antidepressant action (Li et al., 2001; Knapp et al., 2002), promote
serotonin release in the pre-frontal cortex (Ge et al., 2001), and have been implicated
to be of therapeutic benefit in the treatment of schizophrenia (Marenco et al., 2002;
Coyle et al., 2002). Interestingly, glutamatergic inputs also regulate neurotrophin
expression in the cortical telencephalon. Neurotrophins have been linked with anti-
depressant action (Siuciak et al., 1997; Mackowiack et al., 2002) and Ampakines
importantly have been shown to increase levels of certain neurotrophins, specifically
brain-derived neurotrophic factor (BDNF), in the CNS (Lauterborn et al., 2000 & 2003; Legutko et al., 2001; Mackowiak et al., 2002). These compounds are also active in behavioural models predictive of potential anti-psychotic action, such as the amphetamine-induced locomotion test (Johnson et al., 1999), and in behaviour models of anxiety, such as the marble-burying task (Organon personal communication).

When considering the actions of drugs which impact on such a pervasive neurotransmitter system (glutamate), it is also important to consider the effects of functional activation on complex neural networks. Ampakines have been indicated to have greater effects on complex neural pathways as opposed to monosynaptic synapse. This is significant, as many neuropsychiatric and affective disorders, such as depression and schizophrenia, are now believed to be due to problems not in isolated neurotransmitter systems, but in processing within complex polysynaptic networks (‘network’ theory). It is therefore imperative to examine the effects of Ampakines in whole systems with regard to functional activity, to attempt to dissect their possible effects in brain regions and circuitry relevant to disease states. It is exactly this point that this thesis will address by combining use of the novel Ampakines Org 26576 and Org 24448 with functional brain imaging (see below section 1.9.).

Clinically therefore, Ampakines may provide a novel means by which subtle modulation of receptor function results in the subsequent enhancement of synaptic function improving cognitive output and alleviating deficits in disease states associated with deficient glutamatergic neurotransmission, without affecting overall basal transmission, or the induction of excitotoxic effects.

1.8. AMPA Receptor Distribution in the CNS

It is essential when considering the effects of drugs, particularly ones that act on specific receptors, to identify in the CNS where the compounds are most likely to act. For example in this thesis, where the effect of the Ampakines Org 26576 and
Org 24448 will be investigated, it is important to identify the distribution of AMPA receptors, to allow any functional changes induced by the Ampakines to be correlated with the presence or absence of AMPA receptors.

The exact distribution of AMPA receptors in the CNS was initially characterised in the mid-1980s by several groups employing $[^3]H$-AMPA autoradiographic binding studies in the rat (Halpain et al., 1984; Rainbow et al., 1984; Monaghan et al., 1984; Olsen et al., 1987). These studies identified that AMPA receptors were heterogeneously distributed across the CNS, with binding largely restricted to telencephalic structures. Particularly high levels of binding were evident in the hippocampus, cerebral cortex, septum and striatum. The hippocampus displayed regional variations, with the highest levels seen particularly in the CA1 pyramidal layer and dentate gyrus molecular layer. Binding was also high in the CA2 and CA3 pyramidal layers. Binding in the cortex was laminar in nature with high levels in the inner cortex and lower levels in the middle and deep cortex. Visual cortex, somatosensory cortex, caudate putamen, nucleus accumbens, basal ganglia and the amygdala all displayed moderate levels of binding; and low levels were evident in the thalamic nuclei, midbrain, hypothalamus, cerebellum, and brain stem. The lowest level of binding was evident in the globus pallidus. Following the cloning of the AMPA receptor subunits, in situ hybridisation for mRNA transcripts and immunohistochemistry to detect the protein itself, were used to further investigate AMPA distribution in the CNS. The results of these studies were consistent with the findings obtained by ligand-binding, with high levels of immunolabelling detected in the hippocampus, septum, cerebral cortex, habenula, amygdala and lower levels in the cerebellum (Petralia and Wenthold, 1992; Martin et al., 1993). There were however profound regional differences in the pattern of immunostaining for the individual subunits, suggestive that there is a large possible diversity in subunit combinations expressed by neurones.

It should also be observed that AMPA receptors are also found on glial cells (Gallo and Russell, 1995; Garcia-Barcina and Matute, 1998; Janssens and Lesage, 2001). A recent study demonstrated that mouse hippocampal astrocytes can be categorized
into AMPA receptor expressing cells and glutamate transporter cells (Wallraff et al., 2004). Functionally, glia play an essential role as an energetic shuttle between the blood supply and neurons (Yoshioka et al., 1996).

1.9. Imaging the Effects of Drugs in the CNS

As described previously, this thesis will investigate how the novel Ampakines Org 26576 and Org 24448 affect overall functional activity in the CNS. Traditionally, receptors and the actions of pre-clinical drugs on said receptors were, and still are, investigated in the CNS using largely indirect pharmacological and molecular imaging methodologies such as autoradiographic binding, in situ analysis, and c-fos expression studies.

The classical pharmacological technique of autoradiographic binding for example utilises radiolabeled ligands to determine the tissue distributions of receptors and the action of compounds upon them. For example, a recent study with the tritiated AMPA receptor potentiator LY395153 in 2004 identified that it bound to rat tissue sections with 85% specific binding, displaying high levels of binding in the hippocampus (CA1 and CA3), and cortex (layers I-III) (Weiss et al., 2004). Low levels of binding were observed in the globus pallidus and geniculate nuclei, results which correspond closely with the AMPA receptor distribution outlined above (Section 1.8.).

*In situ* hybridisation employs oligonucleotide probes designed specifically against the targets of interest (i.e. GluR1-4), which are labelled (radioactively or fluorescently) for easy identification. This methodology allows the pattern of expression of receptors, via their RNA message (mRNA), either native or induced by drugs, to be identified. In a similar manner, c-fos expression studies image the immediate early gene *c-fos* as a marker of neuronal activity. Both these techniques are indirect, and even though they provide valuable and undisputable insights into understanding receptor distribution and under certain circumstances function, they only provide a certain percentage of the overall picture.
Fig 1.10. Representative images of traditional forms of imaging receptor
distribution

(A) Autoradiograph of the tritiated AMPA ligand Ro48 8587 binding to AMPA
receptors at the levels of the hippocampus. (B) Autoradiograph of $^{35}$S-labelled
oligonucleotide probes specific for GluR2 mRNA in a horizontal section of a mouse
brain (hippocampus is indicated by arrow).

Another issue with the above-described methodologies is that they only allow for the
identification of specific regional changes in binding/expression. With investigations
into compounds that are not specific but affect widespread systems, such as the
glutamatergic system, it is also essential to attempt to identify changes in complex
polysynaptic circuitry, to glean some insight into their overall effects.

The $[^{14}]$C-2-deoxyglucose autoradiographic technique, first introduced by Sokoloff
in 1977, is a method that affords both a novel and unique way to map complex
functional neural pathways simultaneously in all anatomical components of the CNS
(Sokoloff, 1977), in isolated animals or in response to pharmacological intervention.
The proposed model was based on one key physiological principle: that in normal
aerobic conditions the brain derives its energy from the catabolism of glucose,
through a well-defined series of enzymatic reactions (see Methodology). As function
is intimately related to energy consumption, by determining glucose utilisation
within a structure it is possible to accurately estimate the level of functional activity
within that same structure. Therefore, this technique not only allows the
identification of localised changes in cerebral metabolism, but also since alterations
in glucose use correspond to activity in neuronal pathways it allows the investigator
insight into activated polysynaptic circuitry in the cerebrum (most commonly used in
response to drug administration).
Indeed the $[^{14}\text{C}]-2$-deoxyglucose autoradiographic technique has been used extensively to characterise the circuitry associated with multiple neurotransmitter systems in the CNS, primarily through the administration of agonists, antagonists or strategically placed lesions to disrupt circuitry. It is this technique that will be employed in this thesis to examine the effects of the novel Ampakines Org 26576 and Org 24448 on functional activation and circuitry in the mouse cerebrum, which will provide valuable insights in addition to techniques such as in situ hybridisation and classical autoradiography to hopefully provide a ‘fuller’ picture of how these compounds elicit their effect in the CNS.

**1.10. Thesis Aims**

The specific aims of this thesis were:

1. To establish a mouse model of semi-quantitative $[^{14}\text{C}]-2$-deoxyglucose autoradiographic imaging, and to validate this model both by comparison with previously published data and through the use of well-characterised pharmacological compounds.

2. To determine the anatomical site of action of the novel Ampakines Org 26576 and Org 24448 utilising the established semi-quantitative $[^{14}\text{C}]-2$-deoxyglucose autoradiographic imaging technique.

3. To establish that effects with these novel Org Ampakines on cerebral activation are indeed directly AMPA receptor mediated effects.

4. To determine the effects of chronic administration of the novel Ampakines Org 26576 and Org 24448 on cerebral function utilising the established semi-quantitative $[^{14}\text{C}]-2$-deoxyglucose autoradiographic imaging technique.

5. To determine the effects of chronic administration of the novel Ampakines Org 26576 and Org 24448 on neurogenesis, AMPA receptor expression and intracellular signalling in the murine hippocampus.
6. To determine the ability of the novel Org Ampakines Org 26576 and Org 24448 to modify both baseline kinetic properties of AMPA receptors and a paradigm of synaptic plasticity, long-term-potentiation (LTP), in the murine hippocampus.
Chapter 2

Materials and Methods
2.1. Animals

All procedures were carried out under licence from the Home Office with the approval of the University of Edinburgh Ethical Review Panel and were subject to the Animal (Scientific Procedures) Act 1986. Adult male C57Bl/6J mice were purchased from Charles Rivers Ltd. (Cambridge, UK), and communally housed on a 12hr light/dark cycle with free access to food and water.

2.2. $^{14}$C-2-deoxyglucose Autoradiography

2.2.1. Theory

As mentioned in the introduction, the $^{14}$C-2-deoxyglucose autoradiographic technique was first introduced by Sokoloff in 1977, as a method that afforded both a novel and unique way to map functional neural pathways simultaneously in all anatomical components of the central nervous system. The proposed model was based on one key physiological principle: that in normal aerobic conditions the brain derives its energy from the catabolism of glucose, through a well-defined series of enzymatic reactions. As function is intimately related to energy consumption, by determining glucose utilisation within a structure it is possible to accurately estimate the level of functional activity within that same structure.

The experimental technique purported by Sokoloff is based around the existence of a structural analogue of glucose, 2-deoxy-D-glucose. This analogue differs from native glucose in only one way, specifically; the hydroxyl group on the second carbon of the glucose atom is substituted with a hydrogen (H) (Fig 2.1.). This single structural difference imparts chemical properties on the molecule making it ideally suited for the proposed methodology.

Both glucose and 2-deoxyglucose are transported between the blood and brain tissues across the blood brain barrier by the same saturable carrier. However once in the brain tissue, these two molecules are differentially metabolised (Fig 2.2.). Normal glucose is converted by hexokinase into glucose-6-phosphate, which is then acted upon by phosphohexose isomerase, isomerising it to fructose-6-phosphate. This
Fig 2.1. Diagrammatic representation of the cyclic form of glucose (A) and its analogue 2-deoxyglucose (B)

The substitution of the hydroxyl group on the second carbon atom with a single hydrogen (star) results in a subtle shift of the molecule’s properties, resulting in it being unable to be metabolised by phosphohexose isomerase.

![Diagram of glucose and 2-deoxyglucose](image)

---

**Fig 2.2. A schematic representation of the differential metabolism of glucose and [14C]-2-deoxyglucose in the brain**

\[
\begin{align*}
C_P' / C_P & \quad \text{Concentration of [14C]-2-deoxyglucose and glucose in arterial plasma.} \\
C_E' / C_E & \quad \text{Concentration of [14C]-2-deoxyglucose and glucose in the tissue pools that serve as substrates for hexokinase.} \\
C_M' / C_M & \quad \text{Concentration of [14C]-2-deoxyglucose and glucose in tissue} \\
K'_{1/23} / K_{1/23} & \quad \text{Rate constants for the carrier-mediated transport of [14C]-2-deoxyglucose and glucose respectively from the plasma to the tissue (1) and from the tissue back into the plasma (2), and for the rate of phosphorylation by hexokinase (3) (Sokoloff et al., 1977).}
\end{align*}
\]
product enters the glycolytic and tri-carboxylic acid pathways to generate energy in the form of ATP. 2-deoxyglucose, similarly to glucose, is acted upon by hexokinase converting it into 2-deoxyglucose-6-phosphate. However, unlike normal glucose, metabolism stops at this point as 2-deoxyglucose-6-phosphate is not a valid substrate for phosphohexose isomerase (due to the lack of the OH group). This reaction is virtually irreversible as 2-deoxyglucose-6-phosphate is also not a substrate for other glucose-6-phosphate metabolising enzymes such as glucose-6-phosphatase, coincidentally whose enzymatic activity is very low in mammalian brain tissue. Thus the 2-deoxyglucose-6-phosphate is essentially trapped, and subsequently accumulates in the tissue as it is formed for the duration of the experiment. By substituting the normal C atoms in the glucose molecule with the radioactive β-emitting $^{14}$C atoms, the 2-deoxyglucose-6-phosphate can be visualised once trapped by means of autoradiography.

Sokoloff, also in his 1977 paper, proposed a mathematical representation of the model known as the ‘operational’ equation, in which he defined the variables to be measured and the procedures to be followed for accurate determination of local cerebral glucose use (LCGU). This equation also incorporated all the assumptions and known data about the methodology (Fig 2.3.).

$$R_i = \frac{C^*_i(\tau) - \frac{1}{\Phi} \int_0^\infty \frac{1}{K_m^*} \left( C_p^* / C_p \right) dt - e^{-(k_1 + k_2)\tau} \int_0^\infty \frac{1}{K_m^*} \left( C_p^* / C_p \right) e^{(k_1 + k_2)\tau} dt}{\lambda V_m^* K_m^*}$$

**Fig 2.3. The operational equation of the [$^{14}$C]-2-deoxyglucose method**

The rate of glucose utilisation ($R_i$) in any region of cerebral tissue is calculated using this equation. The lumped constant components are as follows:

1. $1/\Phi$: Reciprocal of $\Phi$ reflects the level of glucose-6-phosphatase activity.
2. $\lambda$: Represents the ratio of distribution volumes for [$^{14}$C]-2-deoxyglucose and glucose in the tissue.
3. $V_m^* / V_m$: Ratio of maximal velocities of phosphorylation of the [$^{14}$C]-2-deoxyglucose and glucose by hexokinase.
4. $K_m^* / K_m$: Ratio of the Michaelis-Menten constants for [$^{14}$C]-2-deoxyglucose and glucose, which represent the kinetic properties of the enzyme hexokinase.
Briefly, the equation states that if $[^{14}\text{C}]-2$-deoxyglucose is introduced into the blood and allowed to circulate for a given time ($\tau$) then the rate of glucose consumption ($R_i$) in any cerebral tissue (i) can be calculated provided that; the total $^{14}\text{C}$ in that tissue ($C_i^{*}$) is measured at time $\tau$, the entire histories of arterial plasma concentrations of $[^{14}\text{C}]-2$-deoxyglucose-6-phosphate and glucose from time 0 to time $\tau$ are determined, and the rate constants $k_1^{*}$, $k_2^{*}$, $k_3^{*}$ and the single lumped constant are known. However, the viability of the above model is dependant on some additional assumptions/conditions, specifically that:

1. $[^{14}\text{C}]-2$-deoxyglucose is present in tracer amounts.
2. The arterial plasma glucose concentrations and the rates of consumption remain constant throughout the duration of the procedure.
3. Glucose metabolism is in a steady state for the entire experimental duration.

The dynamic alterations witnessed in glucose utilisation as a result of this procedure are thought to primarily reflect neuronal nerve terminal electrical activity (Schwartz et al., 1979). The remainder of the energy consumption is most likely used to maintain appropriate ionic gradients and reflect the intimate role of astrocytes in cerebral metabolism (which is closely linked to neuronal activation).

### 2.2.2. $[^{14}\text{C}]-2$-deoxyglucose autoradiography modifications for mouse

In the standard $[^{14}\text{C}]-2$-deoxyglucose procedure, rats are anaesthetised and have both femoral arteries and veins exposed and cannulated. Their hind limbs are immobilised with Plaster of Paris and the animals are allowed to recover for ~2 hr. 50 $\mu$Ci of $[^{14}\text{C}]-2$-deoxyglucose is then injected via one of the venous catheters over a 30 second period. 14 precisely timed arterial blood samples are taken over the 45 min experimental period, and are analysed for glucose and $^{14}\text{C}$ content. This allows a full profile of plasma glucose/$^{14}\text{C}$ to be constructed across the entire experimental period. Using this data in conjunction with the operation equation allows for extremely accurate determination of LCGU.
In $[^{14}\text{C}]$-2-deoxyglucose autoradiography in the mouse, also termed ‘semi-quantitative $[^{14}\text{C}]$-2-deoxyglucose’, only a single terminal torso blood sample is taken, as it is exceedingly difficult to acquire several blood samples from a mouse without inducing hypoglycaemia due to their overall small circulating blood volume (~1.5-2 ml for a 25 g mouse). As a full profile of glucose/$^{14}\text{C}$ is therefore not available, it is necessary to introduce a ‘control’ variable to restrict the potential error from this semi-quantitative modification (Kelly et al., 2002). This ‘control’ is traditionally a region of cerebral grey matter that is notionally unchanged by all experimental interventions. For the technique to be valid in any given experimental setup, a plot of $^{14}\text{C}$ levels in the control region versus the plasma glucose/$^{14}\text{C}$ ratio is necessary, and should elicit a linear relationship. This demonstrates that the technique is viable and that comparisons made between animals and groups are accurate.

### 2.2.3. $[^{14}\text{C}]$-2-deoxyglucose autoradiography

LCGU was measured in conscious free-moving mice using an experimental procedure previously described by Kelly et al. (2002). Freely moving mice (~25 g) were intraperitoneally injected with 5 µCi of $[^{14}\text{C}]$-2-deoxyglucose (specific activity 57.2 mCi/mmol, Sigma, UK) dissolved in 0.4 ml Mulgofen-saline (5%) at a constant rate over a 10 second period. Mice were returned to their cages for 44 min 35 s and observed. At the end of this period each mouse was transferred to a perspex chamber and briefly anaesthetised in a mixture of 4% halothane in 30% Oxygen / 70% Nitric Oxide for 25 s. At exactly 45 min post-isotope injection the mice were decapitated and a terminal blood sample was collected by torso inversion into pre-heparinised glass beakers. This sample was loaded into pre-heparinised 0.5 ml Eppendorf tubes and centrifuged at 10,000 rpm for 2 min. The plasma fraction of the blood was carefully drawn off the spun samples, and stored at 4 °C. This terminal plasma sample was used to determine both $^{14}\text{C}$ and glucose concentrations by liquid scintillation analysis (20 µL) and semi-automated glucose oxidase assay (Beckman Glucose Analyser II, 10 µL) respectively. The brains were removed rapidly and frozen in chilled isopentane (-42 °C) for 5 min. Frozen brains were stored in 7 ml bijou tubes at -20 °C until sectioning (max 1 week). Serial coronal sections (20 µm
thick) were then prepared in a cryostat at -16°C, sampled every 60 µm through the cerebrum (see cutting section 2.5. below for detailed procedure). Brain sections were collected onto heated glass coverslips and dried rapidly on a hotplate at 60°C. Coverslips were then mounted onto standard cards sequentially, and autoradiograms of these sections were produced by exposing them to ¹⁴C-sensitive film (Kodak-Biomax MR-1 film, Sigma, UK), in light tight X-ray cassettes for exactly 5 days with precalibrated methacrylate ¹⁴C standards (Amersham, UK). Films were developed using an automatic x-ray film developer.

2.2.4. Densitometric analysis of [¹⁴C]-2-deoxyglucose autoradiograms

Densitometric analysis of the resultant autoradiograms was carried out on an AIS Image Analyser (Imaging Research, Canada). The developed films were placed on a stable-intensity light box, and the light intensity was adjusted appropriately to provide a blue-red colour when visualised in the transposed colour mode. A flat-field correction was carried out to eliminate any background interference, and a standard curve (Fig 2.4.) was constructed by measuring the absolute relative optical densities (RODs) of known ¹⁴C precalibrated methacrylate standards (film background intensity was corrected by normalising it to zero nCi/g on the standard curve prior to ¹⁴C standard reading). Calibration was adjusted for each film so that all measurements made on that particular film fell in the accurate linear middle section of the standard curve.

Discrete neuroanatomical regions were assessed, chosen based on pre-determined key regions of interest for each study. Analysis was carried out bilaterally in 6 continuous sections, resulting in 12 measurements in all. The size of the sample area was determined in advance, in relation to the relative size of the area of interest. These pre-determined sample sizes were used throughout the analysis. Measured RODs for these areas were converted into a ‘functional measure’, LCGU (nCi/g), via calibration against the above constructed standard curve. LCGU was estimated as the ratio of the ¹⁴C concentration in the region of interest to that of a control region (cerebellar cortical grey matter): tissue ¹⁴C (nCi/g) in regions of interest vs. tissue ¹⁴C (nCi/g) in the control region.
Methacrylate $^{14}$C standards of a known concentration of radioactivity (nCi/g) are calibrated against their optical densities on exposed film, allowing the accurate determination of LCGU in discrete anatomical brain regions.

2.2.5. Statistical analysis of LCGU data

All data were analysed for statistical significance by one-way analysis of variance, followed by Dunnett’s or Bonferroni’s post-hoc analysis, to correct for multiple comparisons between the drug-treated and vehicle control groups.

2.3. Preparation Stages for Investigation of Protein Changes

2.3.1. Saline perfusion

Animals were deeply anesthetised in a perspex box with 4% halothane in 30% Oxygen / 70% Nitric Oxide, and then transferred to a facemask in a fumehood where the halothane was reduced to a maintenance level (3%) for the remainder of the procedure. The mask and the animal’s forelimbs were taped firmly in place, so the animal’s ventral surface was facing upwards. An initial incision was made at the base of the sternum. The skin and hair was cut away to expose the diaphragm, which was also detached to reveal the chest cavity. The rib cage was cut away either side of the heart and all connective tissue was severed to allow free access the heart. The
heart was immobilised and a butterfly needle (22G) attached to a perfusion pump (Harvard Instruments) was inserted at the apex of the left ventricle and advanced into the left atrium. The needle was clamped in place with surgical-clamp scissors, and the right atrium was cut to allow for complete perfusion of the circulatory system (drainage of blood). 20-25 ml of heparinised ice-cold saline (5000 units/100 ml) at a rate of 2.5 ml/min⁻¹ was administered until the perfusate ran clear. The paling of the paws (extremities) and organs such as the liver were also used as a reference to indicate complete efficient perfusion. The head of the animal was removed, the brain carefully isolated and placed in ice-cold saline.

2.3.2. Micro-dissection and tissue preservation
The brain was transferred to a dissecting surface; half a Petri dish embedded in ice covered with saline-saturated filter paper. Using a razor blade the brain was initially hemi-bisected along its midline. Half the brain was immediately frozen in chilled isopentane (-42°C) for 2.5 min, and placed in storage at -20°C. The other half of the brain was turned so that the medial surface (cut surface) was facing up, and was held down with a pair of Graefe forceps whilst another pair was used to peel the neocortex off the brain exposing the hippocampus. Once exposed, the fornix was clipped and the ends of one set of forceps were gently worked beneath the fimbria, rolling the hippocampus out along its longitudinal axis. Gently cut away from the brain, the dissected hippocampus was placed in a 1.5 ml eppendorf tube, labelled, snap frozen in liquid N₂, and stored at -80°C for tissue homogenisation and Western blot analysis.

2.3.3. Section preparation and collection
Hemi-brains were removed from storage at -20 °C, placed in dry ice and transported to the cryostat (Bright Instruments Ltd.). A rotatable cryostat ‘chuck’ was cooled in dry ice and OCT embedding compound (Raymond Lamb) was applied to it and allowed to partially solidify. The brain was carefully removed from its tube and placed, cerebellum down, into the partially solidified OCT so it lay perpendicular to the axis of the chuck. The OCT was allowed to solidify completely, and further OCT was applied up to the level of the cerebellum/cortical boundary. The chuck was fixed in place in the cryostat and allowed to equilibrate to a cutting temperature of
-16 °C. 20 µm serial coronal sections were cut using the cryostat’s anti-roll plate and collected onto poly-l-lysine pre-coated glass slides. Sections were allowed to fully adhere, and the slides were stored in plastic slide boxes (VW Inc.) at -20 °C until used for immunohistochemistry/morphological analysis.

2.4. Immunohistochemistry

2.4.1 Theory
Immunohistochemistry (IHC) is a technique combining anatomical, immunological and biochemical principles for the localisation of antigens of interest in tissue sections using labelled antigen-specific antibodies, which are visualised by markers such as enzymes (e.g. peroxidase) or fluorescent dyes. IHC allows accurate visualisation of both the distribution and localisation of cellular components within cells and/or tissue, and involves 4 key steps (Fig 2.5.).

2.4.2. General procedure
Sections were removed from storage at -20 °C and were allowed to air dry at room temperature for 1 hr prior to use. Slides were placed in standard slide racks and equilibrated in PBS (1x used throughout) for 5 min. This was followed by dehydration in graded alcohols: 70% (2 min), 90% (2 min) and 100% (2x5 min); and the blockage of endogenous peroxidase activity by immersing the sections in 0.5% H₂O₂ in methanol for 30 min. Subsequent to this, sections were rinsed in running tap water for 10 min and again equilibrated in PBS for 5 min at room temperature. Antigen unmasking was then carried out by microwaving the sections at 90 °C for 10 min in citric acid buffer (pH 6.0). Sections were allowed to cool in the buffer for at least 1 hr. Following 2x5 min rinses in PBS, non-specific binding sites were blocked in 0.5% BSA / 10% normal serum made in PBS for 1 hr at room temperature. The block was drained off and replaced with the primary antibody made in the original blocking solution, which was incubated overnight at 4 °C. The following day, sections were allowed to rest at room temperature and subjected to 2x10 min washes in PBS. The secondary biotinylated antibody was made up in PBS and placed on the sections for 1 hr at room temperature; this was followed by 2x10 min washes in PBS.
The 'ABC' immunoperoxidase method of immunohistochemistry utilises enzyme-conjugated antibodies, in the 4 distinct steps above to yield a coloured or luminescent product, which can be visualised with a standard light microscope. This specific reaction was used due to its stability, high-sensitivity, and low background staining.
The avidin biotinylated horseradish peroxidase solution (Vectastain ABC Elite Kit) was made up according to the manufacturers instructions, ~30 min prior to use, and applied to the sections for 1 hr at room temperature. Sections were washed for 2x10 min in PBS, and developed in diaminobenzidine (DAB) for 5 min. DAB was drained off into a 5% Precept disinfectant solution and sections were washed thoroughly in clean running water to remove any remaining DAB. All sections were counterstained briefly with haematoxylin, differentiated in acid alcohol, rinsed in Scott’s tap water, dehydrated in graded alcohols, cleared in xylene and mounted in DPX mountant. Negative controls for immunohistochemistry underwent all the above steps except the primary antibody was omitted.

2.5. Western Blot Analysis of Protein Levels

2.5.1. Tissue homogenisation
Hippocampal tissue samples were removed from the -80°C freezer and weighed. 5x (wt/vol) of homogenisation buffer of the following composition: 10 mM HEPES pH7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.1% (wt/vol) NP-40, 1 mM PMSF, was added to each sample. Homogenisation was manually carried out on ice in 1ml Dounce tissue homogenisers, employing ten vertical up and down strokes with a rotation/grinding motion at the bottom to help break up the tissue. Homogenates were left on ice for 20 min, transferred to 1.5 ml Eppendorf tubes and spun at 14,000 rpm for 10 min at 4°C. The supernatant fractions were carefully removed, to avoid contamination, placed in clean 0.5 ml eppendorf tubes and stored at -80°C.

2.5.2. BCA assay and protein content determination
The BCA protein assay is a technique used to determine accurate concentrations of protein samples. The assay measures the formation of Cu⁺¹ from Cu⁺² by the Biuret complex in alkaline solutions of protein. The BCA reagent forms a complex with the reduced Cu⁺¹, which manifests as a purple colour product and has a strong absorbance at 562 nm. The intensity of the colour is directly proportional to the protein concentration, allowing accurate concentration determination. The assay is
carried out at 37°C or 60°C, as this increases its sensitivity and helps reduce the variation in the response of the assay to protein composition.

Fig 2.6. Bovine serum albumin (BSA) protein standard curve

Increasing concentrations of BSA (μg/ml) directly correlate to increased absorbency at 562nm, allowing accurate determination of tissue sample protein concentrations by linear regression analysis.

A series of protein standards (10-1000 μg/ml) were prepared in duplicate using bovine serum albumin (BSA) of a known concentration by serial dilution. Protein samples were prepared, in triplicate, from the homogenates in a 1:10 or 1:15 dilution (in the original extraction buffer), and together with the standards these samples were reacted with the BCA reagent at 60°C for 30 min. The absorbency of all the samples was measured on a spectrophotometer at 562 nm, and a graph was constructed for the BSA standards (A_{562} vs. Protein_{μg}) (Fig 1.6.). Linear regression analysis was used to determine the protein concentrations of the samples from their averaged absorbencies.

2.5.3. SDS-PAGE

Electrophoresis is the migration of charged molecules in solution in response to an electric field. In sodium-dodecyl-sulphate polyacrylamide-gel-electrophoresis (SDS-PAGE), SDS an anionic detergent denatures proteins by surrounding the polypeptide backbone and in so doing, confers a negative charge to the polypeptide in proportion
to its length. It is however necessary to break the disulphide bonds in the proteins before they adopt the random-coil configuration necessary for separation by size. This is achieved by the addition of 2-mercaptoethanol, ensuring therefore that migration is determined solely by molecular weight and not overall size of charge.

A Hoefer SE 600 Series Gel Electrophoresis Unit (Amersham, UK) was used for SDS-PAGE. The glass plates were thoroughly cleaned with both water and 70% ethanol. One glass plate was laid on a flat surface, two 1.5 mm spacers were placed along its edges and the second glass plate was positioned on top. This gel sandwich was secured with the supplied clamps to finger tightness, ensuring the spacers were accurately aligned with the plates. Two such sandwiches were created and were secured in the casting cradle, a tight seal at their bases created to allow effective gel casting. Sample combs were inserted into the assembled plates and a mark was made ~2 cm below the bottom most edge of the combs. A 10% polyacrylamide ‘resolving’ gel (see appendix for composition) was prepared and slowly pipetted into the assembly, up to the level of the mark. This gel solution was overlaid with 2 ml of SDS-saturated water, to prevent the gel drying out, and allowed to set for 45 min. Once polymerised, this SDS-overlay was removed by rinsing several times with distilled water, the sample comb was inserted, and a 4% polyacrylamide ‘stacking’ gel (see appendix for composition) was prepared and pipetted on top of the resolving gel up to the top of the glass plates. This was allowed to set for 30 min.

80 μg of each protein homogenate was made up to a volume of 40 μl with the appropriate homogenisation buffer, to which an equal volume of Laemelli sample buffer / 2-mercaptoethanol (9:1 ratio) was added to obtain a final sample volume of 80μl. The samples were vortexed thoroughly, heated to >95°C for 5 min, to ensure full protein denaturation, and briefly spun at 1000 rpm to consolidate the sample.

Once set, the combs were carefully removed from the top of the gel, and the sample wells were washed with 1x running buffer to remove any remaining unset gel / debris. The gel sandwiches were attached to the upper buffer chamber, and removed from the casting apparatus. The upper chamber was inserted into the gel tank, along
with the cooling apparatus, and each chamber was filled to the appropriate levels with 1x running buffer. Prepared protein samples were carefully pipetted into the stacking gel wells using special gel loading tips, along with Kaleidoscope broad range colour markers (Biorad) and predetermined molecular weight standards (Biorad). The lid was placed on the assembly, connected to the power supply and ran overnight (~15 hr) at 50 mA.

2.5.4. Western blotting

Western blotting was carried out in a Hoefer Transphor TE42 Electrophoresis Unit (Amersham, UK). Once fully ran out, the gels were removed from the glass plates, the stacking gel was cut away using a razor blade. The correct orientations of the gels were recorded by cutting away the top left hand corner. Gels were then soaked in transfer buffer for 5 min. Four pieces of western blotting paper and four 3 mm sponges were also soaked in transfer buffer until saturated, and polyvinylidene fluoride (PVDF) membrane of the correct size was soaked in methanol for 2-5 min prior to use, to ensure complete membrane activation. The transfer stacks were assembled as outlined in Fig 1.7., so that the protein molecules would migrate from the gel toward the PVDF membrane (from cathode (black) to anode (grey)). Bubbles were removed from the assembled sandwich by rolling with a glass pipette. The assembled cassettes were quickly inserted into the transfer tank, to avoid draining the sponges, and the transfer buffer level was corrected to fall between the max and min buffer lines. The entire assembly was continually stirred for the duration of the transfer. Transfer was carried out at 4°C at a constant voltage of 75 V for 2 hrs.

Post-transfer, the PVDF membrane now with the transferred, fixed proteins, was removed from the transfer sandwich, marked for reference, and placed in 50ml centrifuge tubes with the protein side facing inwards. The membrane was initially rinsed in 20 mls 1x PBS/Tween (0.1%) for 10 min, followed by the blocking of non-specific binding sites in 1xPBS/Tween (0.1%) containing 10% non-fat milk (Marvel) for 2 hr at room temperature. Blots were then incubated with the appropriate primary antibodies made in 1x PBS/Tween (0.1%) containing 5% non-fat milk overnight at 4°C (Table 2.1.). The following day, blots were rinsed in PBS/Tween (0.1%) for
3x10 min, and incubated in the appropriate secondary antibody also made in 5% non-fat milk in 1x PBS/Tween (0.1%) at room temperature for 2 hr (Table 1.1.). Following this, blots were again rinsed in PBS/Tween (0.1%) for 3x10 min. All incubation steps were carried out on a rotary shaker to ensure equal and consistent distribution of fluid across the blot’s surface. Following the final rinse, blots were removed from the tubes and placed protein-side facing up in a flat dish. Antibody detection was accomplished using ECL (Amersham) pipetted onto the blot’s surface for exactly 1 min. The ECL was drained off on to blotting paper, and the blot was wrapped in Clingfilm, secured in a light tight cassette and exposed in safelight conditions to ECL-sensitive X-ray film for 2 s - 5 min depending on requirements. Exposed film was developed in an automatic x-ray film processor.

Fig 2.7. Western blotting transfer assembly

The transfer sandwich was assembled in a tray containing transfer buffer ~3cm deep, in the order illustrated above to ensure correct migration of the proteins from the gel to the PVDF membrane. All components were appropriately pre-soaked, and bubbles were removed by gentle rolling with a glass Pasteur pipette.

2.5.5. Coomassie blue staining

Following protein transfer the gels were checked for correct migration of proteins, efficiency of transfer, and correct/equal loading of proteins by coomassie blue staining. Gels were covered in a 0.1% coomassie blue in 50% methanol / 5% acetic acid solution for 2 hr on a shaker at room temperature. Gels were de-stained in a 40% methanol and 10% glacial acetic acid solution overnight, to allow accurate visualisation of protein banding.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Manufacturer Code</th>
<th>Dilution</th>
<th>Made In</th>
<th>Secondary Antibody</th>
<th>Manufacturer Code</th>
<th>Made In</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluR1</td>
<td>Chemicon AB1504</td>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GluR2/3</td>
<td>Chemicon AB1506</td>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calbindin</td>
<td>Upstate AB25085</td>
<td>1:250</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td>Upstate 06-182</td>
<td>1:1000</td>
<td>5% Marvel PBS/Tween</td>
<td>HRP-Anti-Rabbit</td>
<td>Amersham Biosciences NA934</td>
<td>5% Marvel PBS/Tween</td>
<td>1:250</td>
</tr>
<tr>
<td>P-ERK</td>
<td>Upstate 07-467</td>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Lyn-Kinase</td>
<td>Santa Cruz SC15</td>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>Sigma A4700</td>
<td>1:1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-CREB</td>
<td>Upstate AB3442</td>
<td>1:500</td>
<td>5% Marvel TBS/Tween</td>
<td>HRP-Anti-Mouse</td>
<td>Amersham Biosciences NXA931</td>
<td>5% Marvel TBS/Tween</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.1. Details of primary and secondary antibodies and relevant solutions used for antigen detection on Western blots
2.5.6. Western blot quantification and statistical analysis

Developed film was visualised on a light box and bands were semi-quantified by measuring their RODs using an AIS Image Analyser (Imaging Research, Canada) system. Antibody bands were quantified in relation to a reference band to control for loading, which unless otherwise stated was actin, and each blot was repeated at least once to ensure validity. Data were analysed for statistical significance by one-way analysis of variance, followed by Dunnett’s or Bonferroni’s post-hoc analysis, to correct for multiple comparisons between the drug-treated and vehicle control groups.

2.6. Extracellular Field Electrophysiology

Simply, electrophysiology is the study of the electrical properties of both cells and tissues. It involves both measuring the voltage differences across cell membranes, and investigating how the flow of electrical current across membranes is regulated. Extracellular field electrophysiology is the study of the collective activity of a many cells in a certain area, hence ‘field’. The simultaneous activation of many neurones (induced in this thesis by a ‘stimulating’ electrode) induces synaptic transmission and the generation of a field potential, which is detected through the use of a ‘recording’ electrode placed in a selected area of interest. Extracellular field electrophysiology is most commonly carried out in vitro in specially prepared brain slices of the tissue of interest. Acute brain slices from the hippocampus were utilised in this thesis.

2.6.1. Solution preparation

The composition of artificial cerebrospinal fluid (aCSF) for recording extracellular electrophysiological responses was obtained from Arai et al. (2004), and is shown in Table 2.2. The solution in which the hippocampal slices were cut was modified by altering the MgSO$_4$ concentration to 2.5 mM. The high Mg$^{2+}$ content of the cutting solution helped prevent excitotoxic cell death from excess glutamate released during tissue sectioning, through the blockade of NMDA receptors.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>F.W</th>
<th>Supplier</th>
<th>(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>Fisher (UK) S/3160/60</td>
<td>124</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
<td>Fisher (UK) P/4280/53</td>
<td>3</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>84.01</td>
<td>Fisher (UK) S/4240/53</td>
<td>26</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>156.01</td>
<td>BDH (UK) 30716</td>
<td>1.25</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>110.98</td>
<td>Fisher (UK) SH3028901</td>
<td>3.4</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>246.48</td>
<td>Fisher (UK) M/1050/53</td>
<td>1.3 (2.5)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>180.16</td>
<td>Fisher (UK) G/0450/53</td>
<td>10</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 2.2. Composition of the extracellular artificial cerebrospinal fluid (aCSF)

The concentration of MgSO₄ in the aCSF was raised to 2.5 mM during the cutting of acute hippocampal slices to help prevent excitotoxic cell death.

2.6.2. Acute hippocampal slice preparation

Mice were deeply anaesthetised in 4% halothane in 30% Oxygen / 70% Nitric Oxide. They were decapitated at the level of the brainstem with ‘standard pattern surgical scissors’ (FST 14000-16, Germany). The skin and hair were subsequently removed with ‘iris scissors’ (FST 14060-09, Germany) to reveal the skull. Using the iris scissors the skull was removed, initially by an incision from the top of the brainstem along the midline of the skull in a posterior to anterior direction in the superior aspect to the point where the cerebellum joins the main cortical body. This section of skull was carefully peeled away using ‘Graefe forceps’ (FST 11052-10, Germany), whilst also taking care to cut away the meninges to prevent damage to the brain. Following this, a further incision was made along the midline of the skull in the superior aspect up to the level of the olfactory bulbs. The remaining skull was peeled away, and the brain was levered out of the remaining cranium using a small flat weighing spatula,
into ice-cold (<4 °C) carbogenated (95%O₂/5% CO₂ (BOC gas, UK)) cutting aCSF. The brain was allowed to cool for 30 s; this not only helped to slow the metabolism of the tissue, minimising damage due to anoxia, but also helped ‘firm’ the brain for sectioning.

The isolated brain was then placed on a cold, wet, flat absorbent surface (aCSF soaked filter paper on a circular glass plate) for dissection. An initial cut (1) was made diagonally through the rostro-caudal extent of one hemisphere using a sharp razor blade to provide a smooth fixing surface. The cerebellum was sectioned away at the hindbrain junction (2) with a coronal cut, followed similarly by the frontal cortex (3). The lateral surface of the remaining hemisphere was then cut away to provide an initial cutting surface for the vibroslicer (Fig 2.8.).

![Fig 2.8. Diagram highlighting the procedure for the preparation of the tissue block for vibrotome sectioning](image)

This dissected tissue block was mounted in place against a silicone rubber block on a Teflon block using cyanoacrylate glue. This block was subsequently secured in place in a Camden Instruments Vibroslicer (Campden Instruments, Loughborough, UK). The cutting chamber was filled with ice-cold carbogenated cutting aCSF for the entire slicing procedure. 400 μm sagittal brain slices were prepared and the hippocampi roughly dissected out using an artists No. 2 paintbrush and spring-loaded dissecting scissors. After dissection, the slices were carefully picked up on a large flat spatula and transferred to a holding chamber (Fig 2.9.) containing continually carbogenated aCSF at room temperature. Slices were incubated for at least 1 hr prior to recording.
Fig 2.9. A pictorial representation of the slice holder and slice chamber

The slice holder (A) was comprised of 1 cm long cut cylindrical segments of a plastic 5 ml syringe super-glued together over a taut nylon grid. The holder was wedged in place in a 250 ml beaker (B) against a segment cut from a 50 ml syringe. The aCSF was constantly carbogenated.

2.6.3. Recording electrode preparation

Extracellular recording electrodes were pulled on a PP83 vertical puller (Narishige, Japan), using a two-stage pull (Stage 1 – 11.2; Stage 2 – 10.0), from filamented borosilicate capillary glass of 1.5 mm external diameter (Intrafil 01-019-06, UK). Electrodes (~2 μm tip diameter) had a final tip resistance of 2-5 MΩ, and were filled with recording aCSF prior to use.

2.6.4. Electrophysiologica]l recording

A single slice was transferred from the holding chamber to the submerged recording chamber (well volume 1.5 ml) (Madison et al., 1991). The submerged recording chamber was made from a machined perspex block (Fig 2.10.). The aCSF entered the block at the base where it travelled through small diameter glass tubing through a heated reservoir of distilled water, entering the recording chamber through small diameter plastic tubing into an initial bubble trap. Two strips of silicone rubber ran along the sides of the bath upon which fine permeable plastic netting was superglued in place. This acted as a platform upon which the slice sat. The slice was held in place using a U-shaped platinum weight (400 μm thickness), across which, fine elasticated nylon threads were superglued to form a mesh, effectively immobilising the slice. This U-shaped holder was held in place with fine platinum weights at each
corner, and the aCSF level was controlled via a suction tube, connected to a vacuum pump, placed in the rear well of the chamber. Altering the level of this suction device allowed not only flow of aCSF across both surfaces of the slice (hence ‘submerged’), but also removed and circulated new gassed aCSF in a continual unidirectional manner via the gravity-fed perfusion system. Flow rate was restricted by narrowed silicone tubing of a specific length, and was subsequently consistently maintained at ~2.5-3 ml/min⁻¹. Temperature was maintained at 33.5°C ± 0.5°C using a thermostatic controller. An Ag/AgCl₂ earth-electrode was also placed in the rear well and earthed through the head stage of the recording electrode. Illumination of the slice was provided by a fibre-optic light-source. The recording chamber, perfusion system, electrodes and micromanipulators were all situated on a pressurised air table, surrounded by a Faraday cage, to help reduce external vibrations and electrical interference (‘noise’).

**Fig 2.10.** The submerged recording chamber utilised for extracellular electrophysiological field recordings

Carbogenated aCSF entered via the gravity fed perfusion system (1) into the reservoir with distilled water where it was heated to 33.5°C, under thermostatic control (5). aCSF entered the bath in the front chamber (2) where it flowed towards the rear chamber (4) over the slice which was placed and secured on the nylon mesh (3). The level of aCSF and was controlled by means of a suction device placed in the rear chamber.
The filled glass-recording electrode was mounted on a 0.1 mV head stage, which was fixed to a micromanipulator (Narishige, Japan), with the tip of the chlorided silver wire in contact with the recording solution. This was visually lowered toward the slice using the gross control mechanisms. The twisted insulated bipolar stainless steel stimulating electrode was also positioned over the slice and visually lowered into place above the slice. The slice was then visualised with a Leica Optical Microscope, and both recording and stimulating electrodes were finely positioned, using the fine controls of the micromanipulators, in the stratum radiatum of the CA1 field of the hippocampus, allowing recording of field Excitatory Post Synaptic Potentials (fEPSP’s) in response to activation of the Schaffer-commissural fibres.

2.6.5. Data acquisition and analysis
An axoclamp 2A amplifier (Axon Instruments, CA, USA) was used to amplify the signals and to inject depolarising current pulses, through the stimulating electrode. A calibration pulse of 0.5mV was recorded prior to the stimulation pulse and used to determine the amplitude of responses achieved. All data were filtered and digitised using an analogue-to-digital converter (1401 Plus, CED), and the timings of all these events was controlled through a computer-linked interface using the Signal data acquisition and analysis system (Ver. 2.15 CED, Cambridge, UK). Extracellular field recordings were visualised during the course of the experiments both on a standard oscilloscope and via Signal on the computer monitor. Data were stored via Signal on the computer hard disk, and intermittently backed up onto flash disk. Data were analysed off-line using Signal, Microsoft Excel and SigmaPlot/Stat. All data were analysed for statistical significance by paired student’s t-tests with the relevant control responses; significance was set at P<0.05.
Chapter 3

Characterisation and validation of an *in vivo* semi-quantitative model of $^{14}$C-2-deoxyglucose autoradiography in the C57Bl/6J mouse
3.1. Introduction

The $[14^C]$-2-deoxyglucose technique has been used extensively since its inception in 1977 (see Methodology 2.2.1.). Proposed initially in the rat, the methodology has been used extensively to map profiles of activation in response to pharmacological intervention, as well as physiological and pathological states in rodents and higher mammals. Diverse phenomena ranging from the areas of the brain displaying altered function following cortical/hippocampal lesions (Reinstein et al., 1979), to the effects of psychotropic compounds such as cocaine, PCP, and ecstasy on central cerebral function have been investigated (Sharkey et al., 1991; Meibach et al., 1979). Though it has been employed extensively in rats, the technique has limited use in other rodents such as the mouse, due to what is viewed by many as a ‘compromisation’ of the original model purported by Sokoloff. Indeed, papers began appearing in the literature as early as 1980 regarding possible modifications of the methodology, such as intraperitoneal versus intravenous administration of the $[14^C]$-2-deoxyglucose (Meibach et al., 1980), to allow the technique to be successfully translated into mice. With advances in molecular biology, particularly with the generation of transgenic and knockout mice, offering novel avenues for investigation, the use of mice as opposed to rats has been the focus in recent years as an experimental tool. As a consequence of this, various groups have recently used both semi-quantitative and fully quantitative models of mouse $[14^C]$-2-deoxyglucose to investigate functional alterations in glucose use in response to neuropharmacological agents, such as ketamine and MK-801, in normal and transgenic animals (Miyamoto et al., 2000; Kelly et al., 2002; Duncan et al., 2002). These models have proved successful and have elicited responses similar to those witnessed in the rat model.

3.1.1. Aims

The primary aim of this study was to establish an accurate and reproducible model of functional $[14^C]$-2-deoxyglucose autoradiographic imaging in the mouse. In addition to this, it was aimed to further characterise and validate this model with the well-researched compounds (+)-5-methyl-10,11-dihydro-5Hdibenz[a,d]cyclohepten-5,10-imine maleate (MK-801), an NMDA receptor antagonist, and the prototypic Ampakine CX516. As this was a new investigation of Org 26576 and Org 24448 in the mouse, a pilot study was also
carried out utilising a single dose of Org 24448 and Org 26576 to determine whether at these doses, regional alterations in LCGU could be detected and the variability of this response.

3.2. Materials and Methods

3.2.1. Animals
C57Bl/6J mice (25g) were communally housed on a 12hr light/dark cycle for 1 week prior to experimentation with free access to food and water. On the day of experimentation mice were separated into individual cages in the procedure room and allowed to acclimatise to their new environment for 30 min. All experiments were controlled as stringently as possible for weight, light and sound conditions, and circadian timings to ensure equal distribution of variables across the treatment groups.

3.2.2. Drug administration and the [14C]-2-deoxyglucose procedure
Vehicle (5% Mulgofen-Saline (EL-719, GAF Ltd. Manchester, UK)), the NMDA receptor antagonist MK-801 (0.5 mg/kg), the Ampakine CX516 (30 & 100 mg/kg), Org 26576 (3 mg/kg) and Org 24448 (10 mg/kg) were administered intraperitoneally (0.2 ml total volume) 10 min prior to the [14C]-2-deoxyglucose pulse. 5 μCi [14C]-2-deoxyglucose (specific activity 57.2 mCi/mmol, Sigma, UK) dissolved in 0.4 ml vehicle was administered via intraperitoneal injection over a 10 s period in the contra-lateral side of the abdomen to which the drug was injected. The detailed autoradiographic experimental procedure is outlined in section 2.2.3. Standard autoradiographs were prepared and LCGU was determined in pre-determined areas of interest as described in section 2.2.4.

3.2.3. Statistical analysis
Generated LCGU data were analysed for statistical significance by comparison to vehicle treated groups by one-way analysis of variance, followed either by a Dunnett’s post-hoc analysis or post-hoc student’s t-test with an appropriate level of Bonferroni’s correction.
3.3 Results

3.3.1 [\textsuperscript{\textit{14}C}]2-deoxyglucose technique in mouse: control treatment

The aim of the first part of this study was to establish baseline values for cerebral activity (LCGU) in C57/Bl6J mice, as no previous data for this strain of mice was available; and subsequently compare these values with the previously published work in MF1 mice by Kelly \textit{et al.} (2002). To this end, mice were intraperitoneally injected with Mulgofen-Saline (5%) (0.2 ml bolus), 10 min prior to administration of the 5 \textmu Ci [\textsuperscript{14}C]-2-deoxyglucose.

3.3.1.1 Behavioural and baseline physiological parameters

Prior to the experimental procedure mice were generally ambulatory in their cages, displaying normal exploratory behaviour punctuated with periods of intermittent grooming. Following intraperitoneal injection of saline mice exhibited no obvious or abnormal changes in behaviour, as expected. Following intraperitoneal injection of 5 \textmu Ci [\textsuperscript{14}C]-2-deoxyglucose the mice displayed a period of quiet inactivity in which they hid under the closed portion of the cage. Ten minutes post-injection however, their behaviour returned to normal, as outlined above, for the remainder of the experimental period. This transient lull in activity was probably a result of shock induced by the intraperitoneal injection.

The baseline physiological parameters: mean terminal plasma glucose and terminal \textsuperscript{14}C concentrations (mM and nCi/g respectively), for both saline-injected C57l/6J mice and saline-injected WT (MF1) mice (Kelly \textit{et al.}, 2002) are presented in Table 3.1. The values for both terminal glucose and \textsuperscript{14}C concentrations are broadly similar. However, the C57Bl/6J display slightly elevated values in comparison to the MF1 mice. In individual animals, \textsuperscript{14}C levels in the cerebellar cortex, the ‘control’ region, correlated well with the individual plasma \textsuperscript{14}C/glucose ratios, eliciting a linear relationship (Fig 3.1.).

3.3.1.2 LCGU and comparison with Kelly \textit{et al.} (2002)

The effects of saline administration on LCGU compared with the published effects in Kelly \textit{et al.} (2002) are presented in Table 3.2. LCGU data in saline treated C57Bl/6J mice
Table 3.1. Baseline physiological parameters for saline intraperitoneally injected C57Bl/6J mice compared with saline injected MF1 mice (Kelly et al., 2002)

Terminal plasma glucose concentrations (mM), and terminal plasma $^{14}$C concentrations (nCi/g) are shown in summary. Values for C57Bl/6J mice are higher than for MF1 mice. Data are presented as mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6J</th>
<th>Kelly MF1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=6)</td>
<td>10.9 ± 0.75</td>
<td>8.7 ± 0.88</td>
</tr>
<tr>
<td>Terminal plasma $^{14}$C (nCi/g)</td>
<td>38.8 ± 5.04</td>
<td>28.9 ± 4.4</td>
</tr>
</tbody>
</table>

Fig 3.1. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for saline injected C57Bl/6J mice

The relationship is suggestive that higher brain levels of $^{14}$C, as measured in the cerebellar cortex, correspond in a linear fashion with an increased terminal blood levels, as related in the $^{14}$C/glucose ratio.
Table 3.2. LCGU data in C57Bl/6J mice following intraperitoneal injection of Mulgofen-Saline (5%) compared to saline-injected MF1 mice (Kelly et al., 2002)

LCGU in C57Bl/6J mice is on the whole higher than that displayed by MF1 mice. However, the hierarchy of brain region functional activation is well preserved and comparable between both groups. Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C levels in the cerebellar cortex (nCi/g) ± S.D.
Fig 3.2. Schematic illustrations of six classical levels sampled throughout the mouse cerebrum (with Bregma coordinates), and their corresponding representative [¹⁴C]-2-deoxyglucose autoradiograms.

is broadly comparable to the data obtained by Kelly for saline treated MF1 mice in the 16 regions examined (Fig 3.2. – example autoradiograms). The LCGU values obtained for C57Bl/6J mice are on the whole higher than Kelly’s, however the hierarchy of brain regions is well preserved. Areas such as the dentate gyrus, known to display low levels of LCGU, and cortical areas, such as the auditory and somatosensory cortex, known to have higher levels of glucose use, all correlate well between both experimental groups.

3.3.2 Validation and investigation with MK-801 and CX516

The effects of two well-characterised drugs, MK-801, an NMDA receptor antagonist, and CX516, the prototypic Ampakine, on LCGU in the mouse brain were examined. This enabled further validation and characterisation of this mouse semi-quantitative [¹⁴C]-2-deoxyglucose autoradiographic model.

3.3.2.1 Behavioural and baseline physiological parameters

All mice intraperitoneally injected with either MK-801 (0.5 mg/kg) or CX516 (100 mg/kg) displayed profound and overt behavioural changes. MK-801 (0.5 mg/kg) treated mice exhibited notably increased motor activity and excitability, with repetitive movements of the head and a side-to-side swaying gait. CX516 (100 mg/kg) treated mice on the other hand, displayed sedative-like effects, characterised by a total lack of activity. None of the mice intraperitoneally injected with saline or CX516 (30 mg/kg) displayed any abnormal or overt changes in behaviour, remaining alert and lively for the duration of the experimental period.

Mean terminal plasma [¹⁴C] and glucose concentrations for all groups did not differ from the mean plasma [¹⁴C] and glucose concentrations for the vehicle mice, with one exception. The mean terminal glucose concentration for the MK-801 (0.5 mg/kg) treated mice was significantly elevated when compared with the saline injected mice (Fig 3.3.). This elevated glucose, which is characteristic of MK-801 administration, was due most probably to the increased motor activity and excitability induced by the compound. Mean [¹⁴C] levels in the cerebellar cortex did not differ significantly between the four treatment groups (Fig 3.4.A). In individual animals [¹⁴C] levels in
Terminal plasma glucose concentrations (mM) (A), and terminal plasma $^{14}$C concentrations (nCi/g) (B) are shown separately and in summary (C). There was no significant difference between either the terminal plasma $^{14}$C and glucose concentrations in all 4-treatment groups, with one exception. Plasma glucose was significantly elevated in mice receiving MK-801 (0.5 mg/kg). Data are expressed as mean ± S.D. *P<0.05, **P<0.01 for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for vehicle (Mulgoen-Saline), MK-801 (0.5 mg/kg) or CX516 (30 & 100 mg/kg) treated C57BL/6J mice

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The ‘control’ relationship (B) was similar between vehicle, MK-801 (0.5 mg/kg) and CX516 (30 mg/kg) groups. The relationship was dramatically altered however with CX516 (100 mg/kg) treatment, to such an extent to indicate non-conformity and invalidate the cerebellar cortex as an appropriate ‘control’ region for this dose of CX516. Data are expressed as mean ± S.D (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
the cerebellar cortex grey matter correlated well with individual plasma $^{14}\text{C}/\text{glucose}$ ratios irrespective of drug treatment, again with one exception (Fig 3.4.B). Mice treated with CX516 (100 mg/kg) did not exhibit the expected linear relationship between $^{14}\text{C} \, (\text{nCi/g})$ in regions of interest vs. $^{14}\text{C} \, (\text{nCi/g})$ in the control region. This data is indicative that MK-801 (0.5 mg/kg) and CX516 (30 mg/kg) did not alter glucose utilisation in the cerebellar cortex, justifying it as an appropriate reference region to control for the semi-quantitative effects with these compounds.

### 3.3.2.2 Effect of the NMDA receptor antagonist MK-801 on LCGU

MK-801 (0.5 mg/kg) elicited widespread alterations in LCGU compared with saline (Table 3.3.). Of the 29 brain regions measured, 24 exhibited significant heterogeneous increases in LCGU, mainly in areas associated with the limbic system, such as the hippocampus, mamillary body, fornix and the thalamic nuclei (Fig 3.5.). LCGU was significantly decreased in one area, the sensory motor cortex. Only three areas, specifically the medial geniculate bodies, lateral habenula nucleus and the inferior colliculus exhibited no significant changes in LCGU with MK-801 when compared with saline administration (Fig 3.6. – example autoradiographs).

### 3.3.2.3 Effect of the prototypic Ampakine CX516 on LCGU

CX516 (30 mg/kg) evoked significant changes in LCGU in only 3 of the 29 areas investigated; specifically, the caudate nucleus, cingulate cortex, and the red nucleus (Table 3.3.). CX516 (100 mg/kg) also elicited minimal changes in LCGU compared to vehicle. When normalised to the ‘control’ region, LCGU significantly differed from LCGU in vehicle animals in only 7 of the 29 areas measured (Fig 3.6.). Significant increases were evident in the anteroventral thalamus, cochlear nucleus, hypothalamus, mamillary body, lateral habenular nucleus and the parasubiculum. One significant decrease in LCGU was evident in the sensory motor cortex with CX516 (100 mg/kg) administration. It was also noted that the autoradiograms displayed a ‘washed out’ appearance, indicative of decreased glucose use throughout the cerebrum.
Table 3.2. LCGU data in C57BL/6J mice following intraperitoneal injection of vehicle (Mulgofen-Saline), MK-801 (0.5 mg/kg) or CX516 (30 & 100 mg/kg)

MK-801 (0.5 mg/kg) had profound effects on LCGU in the mouse cerebrum especially in areas of the limbic system and hippocampus. CX516 (30 and 100 mg/kg) had minimal significant effects on LCGU. Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001 for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Fig 3.5. Effects of MK-801 (0.5 mg/kg) and CX516 (30 & 100 mg/kg) on the hippocampus (A) and the somatosensory cortex (B), compared to vehicle treatment.

MK-801 (0.5 mg/kg) treatment produces significant increases in LCGU in the hippocampus, but results in a significant decrease in glucose use in the somatosensory cortex. Data are presented as mean ± SEM. *P<0.05 (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
MK-801 (0.5 mg/kg) administration results in increases, decreases and no changes in local cerebral glucose use in discrete brain areas when compared to the administration of vehicle. Increases in LCGU are observable in areas such as the hippocampus (H) and Fornix (F); a prominent decrease is evident in the somatosensory cortex (S1); and areas such as the medial geniculate bodies (MG) are unchanged. CX516 (30 mg/kg) administration resulted in minimum changes in LCGU; decreases were evident in the cingulate cortex (Cg1) and the red nucleus (R). CX516 (100 mg/kg) administration resulted in a 'washed out' appearance in the autoradiograms, with what appeared to be profoundly decreased glucose utilisation across the cerebrum. However, when normalised to the control region, LCGU was increased in areas such as the medial habenula nucleus (MHb) and hypothalamus (Hy), with decreases in areas such as the somatosensory cortex (S1), when compared to vehicle administration.

Fig 3.6. Representative autoradiograms illustrating changes in LCGU in specific brain regions in response to administration of MK-801, a potent selective NMDA receptor antagonist, and CX516, the prototypic Ampakine.
3.3.3. **Pilot study of Org 26576 and Org 24448 on functional activity**

Having established the accuracy and reproducibility of a semi-quantitative mouse model of [14C]-2-deoxyglucose, the model was utilised to examine the effects of the novel Ampakines Org 26576 and Org 24448 on cerebral function.

3.3.3.1 **Behavioural and baseline physiological parameters**

All mice treated with vehicle, Org 26576 (3 mg/kg) and Org 24448 (10 mg/kg) displayed no abnormal changes in behaviour. The mean terminal plasma ¹⁴C and glucose concentrations in Org 26576 (3 mg/kg) and Org 24448 (10 mg/kg) treated mice did not differ significantly from mean terminal plasma ¹⁴C and glucose concentrations for the saline treated group (Fig 3.7.). Mean ¹⁴C levels in the cerebellar cortex did not differ significantly in the three groups (Fig 3.8.A), and in individual animals ¹⁴C levels in the cerebellar cortex grey matter correlated well with individual plasma ¹⁴C/glucose ratios, irrespective of treatment (Fig 3.8.B).

3.3.3.2 **Regional alterations in LCGU**

LCGU in Org 26576 (3 mg/kg) treated mice did not in general differ significantly from LCGU in the vehicle treated animals (Tables 3.4-3.6.). Of the 44 brain regions analysed LCGU was significantly increased in only three regions, specifically: the substantia nigra pars reticulata, the caudate nucleus, and the inferior colliculus. However mice treated with Org 24448 (10 mg/kg) displayed widespread homogeneous increases in LCGU compared with vehicle treated mice (Tables 3.4.-3.6.). LCGU was significantly increased in 34 discrete anatomical brain regions of the 44 investigated. The most notable changes were witnessed in the primary auditory and visual systems, components of the limbic system, and to a lesser extent the hippocampus and its associated regions. No significant decreases in LCGU were witnessed with either compound.
Fig 3.7. Baseline physiological parameters in C57Bl/6J mice following intraperitoneal injection of vehicle (Mulgofen-Saline), Org 26576 (3 mg/kg) or Org 24448 (10 mg/kg)

Terminal plasma glucose concentrations (mM) (A), and terminal plasma $^{14}$C concentrations (nCi/g) (B) are shown separately and in summary (C). There was no significant difference between either the terminal plasma $^{14}$C and glucose concentrations in all 4-treatment groups. Data are expressed as mean ± S.D (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Fig 3.8. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for vehicle (Mulgofen-Saline), Org 26576 (3 mg/kg) or Org 24448 (10 mg/kg) treated C57Bl/6J mice

Cerebellar cortex $^{14}$C concentrations (A) for both drug treatments did not differ significantly from vehicle-treated mice. The 'control' relationship (B) was similar between vehicle, Org 26576 (3 mg/kg) and Org 24448 (10 mg/kg) groups. Data are expressed as mean ± S.D (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Vehicle (Mul-Saline)</th>
<th>Org 24448 (10 mg/kg)</th>
<th>Org 26576 (3 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial lateral habenular</td>
<td>1.54 ± 0.07</td>
<td>1.77 ± 0.14*</td>
<td>1.68 ± 0.19</td>
</tr>
<tr>
<td>Fasciculus retroflexus</td>
<td>1.45 ± 0.07</td>
<td>1.87 ± 0.08***</td>
<td>1.45 ± 0.08</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>1.68 ± 0.10</td>
<td>1.92 ± 0.11**</td>
<td>1.72 ± 0.15</td>
</tr>
<tr>
<td>Substantia nigra pars compacta</td>
<td>1.23 ± 0.06</td>
<td>1.40 ± 0.05**</td>
<td>1.32 ± 0.12</td>
</tr>
<tr>
<td>Substantia nigra pars reticulata</td>
<td>0.70 ± 0.04</td>
<td>0.82 ± 0.05**</td>
<td>0.84 ± 0.12*</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>0.99 ± 0.04</td>
<td>1.14 ± 0.09**</td>
<td>1.03 ± 0.06</td>
</tr>
<tr>
<td>Median forebrain bundle</td>
<td>1.25 ± 0.04</td>
<td>1.64 ± 0.11***</td>
<td>1.34 ± 0.12</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.70 ± 0.07</td>
<td>0.79 ± 0.05*</td>
<td>0.77 ± 0.11</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>0.89 ± 0.12</td>
<td>1.20 ± 0.06***</td>
<td>0.94 ± 0.10</td>
</tr>
<tr>
<td>Raphe nucleus</td>
<td>1.21 ± 0.04</td>
<td>1.40 ± 0.03***</td>
<td>1.27 ± 0.06</td>
</tr>
<tr>
<td>Amygdala</td>
<td>0.93 ± 0.04</td>
<td>1.47 ± 0.04***</td>
<td>1.02 ± 0.10</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>1.35 ± 0.08</td>
<td>1.50 ± 0.07*</td>
<td>1.50 ± 0.11*</td>
</tr>
<tr>
<td>Anteroventral thalamic nucleus</td>
<td>1.61 ± 0.08</td>
<td>1.82 ± 0.08**</td>
<td>1.73 ± 0.11</td>
</tr>
<tr>
<td>Laterodorsal thalamic nucleus</td>
<td>1.55 ± 0.06</td>
<td>1.81 ± 0.18*</td>
<td>1.64 ± 0.13</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>1.25 ± 0.03</td>
<td>1.35 ± 0.07*</td>
<td>1.21 ± 0.05</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>1.39 ± 0.09</td>
<td>1.58 ± 0.10*</td>
<td>1.50 ± 0.18</td>
</tr>
<tr>
<td>Fornix</td>
<td>1.01 ± 0.09</td>
<td>1.15 ± 0.09*</td>
<td>1.11 ± 0.16</td>
</tr>
<tr>
<td>Mamillary body</td>
<td>1.91 ± 0.17</td>
<td>2.08 ± 0.14</td>
<td>1.87 ± 0.17</td>
</tr>
</tbody>
</table>

Table 3.4. Effects of Org 26576 (3 mg/kg) and Org 24448 (10 mg/kg) on LCGU in components of the limbic system

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
## Ratio of tissue $^{14}$C (nCi/g) in regions of interest to that in the cerebellar cortex

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Vehicle (Mul-Saline)</th>
<th>Org 24448 (10mg/kg)</th>
<th>Org 26576 (3mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary Auditory System</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>1.68 ± 0.09</td>
<td>2.01 ± 0.14**</td>
<td>1.76 ± 0.15</td>
</tr>
<tr>
<td>Medial geniculate body</td>
<td>1.38 ± 0.06</td>
<td>1.78 ± 0.10***</td>
<td>1.45 ± 0.11</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>2.43 ± 0.04</td>
<td>2.95 ± 0.12***</td>
<td>2.71 ± 0.19*</td>
</tr>
<tr>
<td>Lateral lemniscus</td>
<td>1.37 ± 0.03</td>
<td>1.71 ± 0.07***</td>
<td>1.42 ± 0.12</td>
</tr>
<tr>
<td>Superior olivary body</td>
<td>1.85 ± 0.09</td>
<td>2.05 ± 0.06**</td>
<td>1.73 ± 0.16</td>
</tr>
<tr>
<td>Cochlear nucleus</td>
<td>1.95 ± 0.06</td>
<td>2.30 ± 0.13***</td>
<td>2.06 ± 0.19</td>
</tr>
<tr>
<td><strong>Primary Visual System</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>1.31 ± 0.09</td>
<td>1.45 ± 0.04*</td>
<td>1.38 ± 0.15</td>
</tr>
<tr>
<td>Lateral geniculate body</td>
<td>1.35 ± 0.05</td>
<td>1.55 ± 0.07***</td>
<td>1.45 ± 0.08</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>1.64 ± 0.03</td>
<td>1.65 ± 0.05</td>
<td>1.69 ± 0.06</td>
</tr>
</tbody>
</table>

**Fig 3.5. Effects of Org 26576 and Org 24448 on LCGU in the primary auditory and visual systems**

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Vehicle (Mul-Saline)</th>
<th>Org 24448 (10mg/kg)</th>
<th>Org 26576 (3mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subiculum</td>
<td>1.15 ± 0.06</td>
<td>1.21 ± 0.13</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>1.01 ± 0.05</td>
<td>1.09 ± 0.10</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>1.42 ± 0.08</td>
<td>1.51 ± 0.11</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>Hippocampus - str.molecularae</td>
<td>1.27 ± 0.06</td>
<td>1.37 ± 0.07**</td>
<td>1.24 ± 0.17</td>
</tr>
<tr>
<td>Dentate gyrus - str.molecularae</td>
<td>0.66 ± 0.07</td>
<td>0.81 ± 0.04**</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>Medial septum</td>
<td>1.07 ± 0.07</td>
<td>1.31 ± 0.01***</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Dentate gyrus - dorsal</td>
<td>1.02 ± 0.06</td>
<td>1.08 ± 0.03</td>
<td>1.12 ± 0.10</td>
</tr>
<tr>
<td>Hippocampus - CA1</td>
<td>0.76 ± 0.03</td>
<td>0.76 ± 0.03</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>Hippocampus - CA3</td>
<td>0.84 ± 0.02</td>
<td>0.94 ± 0.01***</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>1.07 ± 0.01</td>
<td>1.11 ± 0.07</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td><strong>Functionally Distinct Regions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red nucleus</td>
<td>1.28 ± 0.07</td>
<td>1.44 ± 0.06**</td>
<td>1.32 ± 0.09</td>
</tr>
<tr>
<td>Inferior olivary body</td>
<td>1.35 ± 0.08</td>
<td>1.66 ± 0.06*</td>
<td>1.32 ± 0.19</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>1.82 ± 0.08</td>
<td>2.02 ± 0.11*</td>
<td>1.97 ± 0.18</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>0.67 ± 0.03</td>
<td>0.71 ± 0.03**</td>
<td>0.75 ± 0.10</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Cerebellar nucleus</td>
<td>1.79 ± 0.06</td>
<td>2.01 ± 0.08**</td>
<td>1.69 ± 0.11</td>
</tr>
</tbody>
</table>

Fig 3.6. Effects of Org 26576 and Org 24448 on LCGU in the hippocampus (associated areas) and functionally distinct regions

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
3.4. Discussion

These results demonstrate that the semi-quantitative model of \textit{in vivo} $^{14}$C-2-deoxyglucose autoradiography in C57Bl/6J mice is both accurate and reproducible. Baseline values obtained are consistently in line with previously published data, and most importantly are reproducible. The model is also able to accurately replicate the effects of a well-characterised compound, the NMDA receptor antagonist MK-801, in respect to functional cerebral changes, and will be predicatively useful in investigating the effects of novel Ampakines, Org 26576 and Org 2448, on cerebral function.

3.4.1. Justification for and establishing a semi-quantitative model of $^{14}$C-2-deoxyglucose in the mouse

Application of the $^{14}$C-2-deoxyglucose autoradiographic procedure in the mouse was first outlined by Nowaczyk and Des Rosier in 1981. They established that values for LCGU in mice were on the whole, lower than previously observed in the rat, and widely varied across the cerebrum, with the highest levels of utilisation in areas involved sensory function, such as hearing, sight and movement.

The technique employed in this thesis, based on Kelly’s protocol (Kelly \textit{et al.}, 2002), varies in two respects from the methodology outlined by Nowaczyk and Des Rosier. Specifically, (1) the $^{14}$C-2-deoxyglucose pulse was administered intraperitoneally instead of via the standard intravenous route, and (2) a single terminal torso blood sample was taken as an alternative to the 14 timed arterial samples throughout the extent of the experimental period. Kelly introduced these modifications to avoid the stress of major surgery and constant blood sampling. They also allowed the animals to undergo the procedure in their ‘natural’ state, fully conscious and freely moving. As a result of these modifications LCGU cannot be measured fully-quantitatively ($\mu$mol/100 g/min), and therefore the data generated is not as precise but does provide robust reliable data, which is highly comparable to the rat.
As outlined previously, the introduction of modifications such as these to Sokoloff’s original technique in the early 1980’s was the subject of much debate and conjecture. Meibach and colleagues in 1980 were the first to introduce the administration of the \([^{14}\text{C}]-2\text{-deoxyglucose}\) via an intraperitoneal route as opposed to intravenous. They reported that whole brain uptake of \([^{14}\text{C}]-2\text{-deoxyglucose}\) at maximal levels of incorporation was the same for both intravenously and intraperitoneally injected animals, and that autoradiograms and their subsequent analysis were indistinguishable and highly correlated. Conversely, Kelly and McCulloch in 1983 reported that intraperitoneal administration resulted in increased levels of un-phosphorylated \([^{14}\text{C}]-2\text{-deoxyglucose}\) in the brain, what they termed ‘a potential source of error’. Kelly et al., (2002) however discovered that the rate of uptake of \([^{14}\text{C}]-2\text{-deoxyglucose}\) in the mouse after intraperitoneal administration was more rapid than described in the rat by Kelly and McCulloch (1983) such that the residual \(^{14}\text{C}\) in cerebellar cortex at the time of sacrifice was \(<~10\%\) of the total \(^{14}\text{C}\) in the region (Kelly et al., 2002). Kelly also argued that this factor, compounded by the fact that there were no significant inter-group differences in terminal plasma glucose, \(^{14}\text{C}\) levels, and cerebellar cortex \(^{14}\text{C}\) levels, results in a technique which does indeed provide a valid estimation of glucose use.

Adding further to the controversy surrounding the validity of these semi-quantitative modifications, Kelly and McCulloch also alleged that semi-quantitative analysis, i.e. the use of optical density ratios, did not yield a constant optical density ratio but was in fact dependant upon the exposure time in the preparation of the autoradiograms and the absolute amounts of \(^{14}\text{C}\) from which the concentration ratio was devised (Kelly and McCulloch, 1983b). One caveat to their criticism however was that ‘…rigorous control of the exposure period and the amount of \([^{14}\text{C}]-2\text{-deoxyglucose}\) administered to each animal might circumvent the difficulties raised by the non-linearity of the relationship between optical density and \(^{14}\text{C}\)-concentrations in cerebral tissue’. This was further supported by Sharpe who reported that optical density ratios were linearly related to LCGU within a given animal (Sharpe et al., 1983), and by Mitchell and Crossman, who argued that this error could be circumvented by ensuring
that films were not approaching saturation when developed (Mitchell and Crossman, 1984).

Simple intraperitoneal injections of saline prior to $[^{14}\text{C}]-2$-deoxyglucose in our hands resulted in no obvious or abnormal changes in behaviour in all mice, eliciting mean terminal plasma glucose and terminal plasma $^{14}\text{C}$ levels comparable to Kelly’s, even if they do appear upon initial inspection to be elevated. In individual animals $^{14}\text{C}$ levels in the cerebellar cortex grey matter correlated well with individual plasma $^{14}\text{C}$/glucose ratios, and LCGU in the saline treated C57Bl/6J mice were broadly comparable to the data obtained by Kelly for saline treated MF1 mice. Values obtained for the C57Bl/6J mice are generally higher, but the hierarchy of brain regions is well preserved, and the errors are comparable in size. The elevations witnessed with the C57Bl/6J mice could be the result of a number of factors, for example the lack of animal handling and \textit{in vivo} experimental experience at this stage, resulting in possible increased stress on the mice. The use of a varied stock of $[^{14}\text{C}]-2$-deoxyglucose isotope compared with Kelly may also have had an effect. These factors may have also been compounded by the fact that there are well-documented strain differences in relation to cerebrovascular structure and metabolism, with regard to MF1 mice, which may conceivably account for the differences in LGCU witnessed between the two groups (Kelly \textit{et al.}, 2001). Overall however, the data is indicative that the semi-quantitative $[^{14}\text{C}]-2$-deoxyglucose technique carried out here is both accurate, and fully in line with previously published data.

\textbf{3.4.2. Model validation with MK-801 and investigation with CX516}

MK-801 is a potent selective and non-competitive NMDA receptor antagonist, first identified as such by Wong and colleagues in the mid-eighties (Wong \textit{et al.}, 1986), which acts by specifically binding to a site located within the NMDA associated ion channel preventing the associated calcium influx. Investigated initially as an effective anti-ischaemic agent in several animal models, the central metabolic effects of MK-801 have been extensively characterised both in mice (Kelly \textit{et al.}, 2002) and in rats (Kurumanji \textit{et al.}, 1989) utilising both semi and fully quantitative $[^{14}\text{C}]-2$-deoxyglucose autoradiography.
MK-801 produces activation resulting in marked heterogeneous and highly characteristic changes in cerebral function. It is useful as a validation tool as it induces a broad spectrum of effects on LCGU, ranging from no effects in some regions to increases and even decreases in others. Effects reported previously in the literature include decreases in LCGU primarily in the inferior colliculus and somatosensory cortex, while prominent increases are evident in major components of the limbic system such as the thalamus, hippocampus, mamillary body, interpenduncular nucleus, and cingulate cortex. Other discrete areas displaying characteristic increases in LCGU include the substantia nigra, cerebellar nuclei, sensory systems, and the fornix, a myelinated fibre tract.

The results of application of MK-801 (0.5 mg/kg) in this semi-quantitative model of 2-deoxyglucose correlated closely with the characteristic responses outlined above. Significant activation was witnessed across the limbic system, LCGU was depressed in the somatosensory cortex, and both the lateral habenula and medial geniculates displayed no alteration in LCGU when compared to vehicle. One exception was that the inferior colliculus remained unchanged instead of demonstrating a depression in LCGU. Another notable ‘irregularity’ was the significant increase in terminal glucose levels witnessed in MK-801 treated mice. This was most probably due to the general increased metabolic excitability induced by MK-801 coupled with the fact that the mice were freely moving and active. The control relationship however was unaffected by this aberration. The ability of this semi-quantitative model of 2-deoxyglucose imaging in the mouse to accurately reflect the characteristic quantitative effects obtained in rats as demonstrated here, has also been verified recently with other compounds such as PCP in both rat quantitative and semi-quantitative [14C]-2-deoxyglucose (Cochran et al., 2005).

The action of CX516 was also investigated in this study along side that of MK-801, to validate the use of this methodology with Ampakines prior to application of the novel compounds Org 26576 and Org 24448. CX516 is considered the prototypic Ampakine compound, and has well demonstrated effects in rodent models of cognition / learning and memory and LTP (Staubli et al., 1994 a/b; Larson et al.,
However in this study, CX516 at both concentrations studied (30, 100 mg/kg) had minimal effects on LCGU. CX516 (30 mg/kg) had minimal effects on LCGU with changes observed in only three areas. LCGU was decreased in the cingulate cortex and red nucleus, and increased in the cerebellar nucleus. This limited response may be due to that fact that CX516 is a first generation Ampakine, and as such, is of low potency and is metabolised and cleared quickly in rodents, subsequently restricting its effects. CX516 (100 mg/kg) on the other hand had unexpected effects in this model. CX516 (100 mg/kg) treatment resulted in alteration in LCGU in only seven regions. LCGU was increased in various limbic structures such as the anteroventral thalamus, mamillary body, and in other ‘sensory’ structures such as the cochlear nucleus and the somatosensory cortex. Furthermore, the resultant autoradiograms appeared ‘washed out’ in appearance and the terminal plasma $^{14}$C levels in the cerebellar cortex failed to correspond in a linear fashion to the terminal plasma $^{14}$C/glucose ratio, hence essentially ‘invalidating’ the methodology for this dosage of CX516. This lack of linearity is due to the elevated levels of terminal plasma $^{14}$C, which in turn resulted in elevations in the plasma $^{14}$C/glucose ratio resulting in a rightward shift in the control plot. This elevated terminal plasma $^{14}$C correlates well with the ‘washed out’ appearance of the autoradiograms as essentially less $^{14}$C has effectively made its way across the blood brain barrier, hence the elevated plasma levels. We are effectively witnessing a ‘deficiency’ of sorts in $^{14}$C transport into the cerebrum with this high dosage of CX516, or possibly an increased rate of conversion of 2-deoxyglucose-6-phosphate to 2-deoxyglucose and retrograde transport from the brain to plasma.

It is also worthy to note that the vehicle data and errors for LCGU from this validation study, were near identical to the LCGU data obtained for the saline injections in the first part of the study, further validating the reproducibility and consistency of this methodology.

### 3.4.3. Org 24448 and Org 26576 pilot study

A pilot study was carried out with the novel Ampakines Org 24448 and Org 26576 to investigate the validity of studying the effects these compounds in this model, and
also to determine an accurate dosing regime and group sizes for a future full dose-response study.

Pilot doses for Org 24448 (10 mg/kg) and Org 26576 (3 mg/kg) were chosen based on effective concentrations in behavioural models predictive of antidepressant action and antipsychotic activity (personal communication Organon). Both compounds produced significant increases in LCGU in the mouse cerebrum, Org 24448 more so than Org 26576. Org 24448 increased LCGU across the mouse cerebrum particularly in the limbic and auditory systems. Considering Ampakines are of potential significance in treating cognition, depression and other affective disorders, the observed limbic system involvement is of great potential importance. In contrast, Org 26576 (3 mg/kg) administration resulted in only 3 significant increases in LCGU, yet did display general increases in other areas which did not reach significance due to large standard deviations within the data set. This once again raises the issue of accurate dosing, and the importance of the group sizes being sufficiently large enough to provide adequate power in a full dose-response study to accurately resolve the true effects of Ampakine drug application.

$$n = \frac{8 \sigma^2}{e^2} = \frac{8(0.055)^2}{(0.065)^2} = 5.7$$

**Fig 3.9. Group size power calculation**

To determine an accurate group size for a full dose response study, a post-hoc power analysis is needed. To accurately determine a significant 5% difference change over baseline (vehicle) with 95% power, an n of 5.7 subjects per treatment group is needed. e = acceptable sampling error (5% in this case of 1.3 = 0.065), \(\sigma\) = standard deviation of the sample (0.055), n = the sample size needed to detect a significant difference. These values and the subsequent calculations carried out upon them were obtained from the variation witnessed between the vehicle and the average change in Org 26576 and Org 24448 data obtained from the pilot study outlined above.

### 3.4.4. Summary

In conclusion, the semi-quantitative \([^{14}C]-2\text{-deoxyglucose}\) model established here in C57Bl/6J mice has been shown to be both consistent and reproducible, as it correlates well with previously published work. It is also accurate, clearly replicating the
classical effects of the NMDA receptor antagonist MK-801 on cerebral function, and will be predicatively useful in investigating the effects of the novel Ampakines, Org 26576 and Org 24448, on local cerebral function.
Chapter 4

Investigation into the effects of *acute* administration of the novel Ampakines Org 26576 and Org 24448 on functional activity in the murine cerebrum
4.1. Introduction

AMPA receptor potentiaters enhance synaptic transmission, by modulating desensitisation and/or deactivation of ionotropic AMPA channels, without directly binding to the glutamate agonist-binding site (see section 1.7.4.). The ‘Ampakines’ class of compounds originated with the development of CX516 in the early 1990s, and as such it is one of the most extensively investigated compounds in its series. Initial experiments with CX516 determined that it did indeed positively modify AMPA receptor kinetics, specifically resulting in a four-fold slowing of deactivation with little effect on desensitisation, and facilitated LTP in vitro (Staubli et al., 1994b). Importantly it was also subsequently established that the compound displayed a lower threshold for effects on complex neural pathways as opposed to simple monosynaptic transmission (Arai et al., 1996). Clinically CX516 improved memory encoding in behavioural assessments in rodents (Staubli et al., 1994b; Hampson et al., 1998), and improved aspects of memory in humans (Ingvar et al., 1997). CX516 has also been investigated as a potential treatment for schizophrenia, both in combination with clozapine and as a single treatment, with promising results (Goff et al., 2001; Marenco et al., 2002).

Org 26576 and Org 24448, the two Ampakine compounds to be investigated in this thesis, are structurally derived from CX516, and have also been shown to have diverse effects both in vivo and in vitro. The compounds increase AMPA currents, are affective in behavioural models of depression and psychoses, induce 5-HT release in the prefrontal cortex (Ge et al., 2001), and are effective in a model predictive of positive cognitive effects (DMTP: delayed-matching-to-position task). However, despite the overall wealth of knowledge on these compounds, little is know about their precise anatomical location of action in vivo.

4.1.1. Aims

The primary aim of this study was to identify the discrete neuroanatomical basis of the action of the novel Ampakines Org 26576 and Org 24448 in the murine cerebrum, to provide insight in to their potential therapeutic relevance, utilising the
established and validated semi-quantitative [$^{14}$C]-2-deoxyglucose autoradiographic methodology (Chapter 3). In order to define whether the effects of Org 26576 and Org 24448 were AMPA receptor mediated, changes in LGCU induced by the Ampakines were also assessed in the presence of pre-administered 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), a selective potent AMPA receptor antagonist.

4.2. Materials and Methods

4.2.1. Animals
C57Bl/6J mice (25 g) were communally housed on a 12hr light/dark cycle for 1 week prior to experimentation with free access to food and water. On the day of experimentation mice were separated into individual cages in the procedure room and allowed to acclimatise to their new environment for 30 min. All experiments carried out were controlled as stringently as possible for weight, light and sound conditions, and circadian timings to ensure equal distribution of variables across the treatment groups.

4.2.2. Drug preparation, administration and the [$^{14}$C]-2-deoxyglucose autoradiographic procedure
The novel Ampakines Org 26576 and Org 24448, and the AMPA receptor antagonist NBQX, were made up as stock solutions prior to the beginning of the study, aliquoted, and stored at -20°C. On the day of experimentation the required volume was defrosted and made up to the final appropriate dosage in a 0.2 ml bolus per mouse. Org 26576 (0.1, 1, 10 mg/kg) and Org 24448 (3, 10, 30 mg/kg) or vehicle (5% Mulgofen-Saline) was administered intraperitoneally ten minutes prior to the administration of the 5 μCi [$^{14}$C]-2-deoxyglucose pulse (doses were chosen on the data produced in the pilot study). All drugs/vehicle were administered in the contralateral side of the abdomen to the [$^{14}$C]-2-deoxyglucose injections. A separate group of mice were treated with the AMPA receptor antagonist NBQX. NBQX (10mg/kg) was injected either alone or 10 minutes prior to Org 26576 (10 mg/kg i.p.), Org 24448 (10 mg/kg i.p.) or vehicle (5% Mulgofen-Saline i.p.). The detailed
autoradiographic experimental procedure is outlined in section 2.2.3. Standard autoradiographs were prepared and LCGU was determined in pre-determined areas of interest as described in section 2.2.4.

4.2.3. Statistical analysis
All data were analysed for statistical significance by one-way analysis of variance, followed by Dunnett’s post-hoc analysis, to correct for multiple comparisons between the drug-treated and control groups.
4.3. Results

4.3.1. Org 26576 and Org 24448 dose-response relationship

4.3.1.1. Effect of Org 26576 and Org 24448 on behavioural and baseline physiological parameters

Org 26576 (0.1, 1, 10 mg/kg) and Org 24448 (3, 10, 30 mg/kg) resulted in alterations in the overt behaviour of the mice only at the highest dose. Vehicle (Mulgofensaline), Org 26576 (0.1, 1 mg/kg) or Org 24448 (3, 10 mg/kg) treated mice did not exhibit changes in their behaviour, as defined using a gross qualitative behavioural analysis. They remained fully conscious, lively and alert throughout the experimental period. However, administration of Org 26576 at 10 mg/kg or Org 24448 at 30 mg/kg was associated with changes in the overt behaviour of mice post-injection. Initially the behaviour of the mice appeared normal post-drug injection for approximately 10 min. However this was followed by a prolonged period of inactivity, immediately following the [14C]-2-deoxyglucose injection, lasting for the remainder of the experiment, during which the mice were quietly hunched in the closed portion of the cage. Despite this lassitude the mice displayed intermittent grooming behaviour, were alert and responsive to stimuli.

Org 26576 and Org 24448 treatment at all doses had no significant effect on the mean terminal plasma 14C and glucose concentrations as compared to the relevant vehicle treated groups (Fig 4.1.). Mean 14C levels in the cerebellar cortex did not differ significantly between the four groups (Fig 4.2.A-4.3.A). In individual mice, 14C levels in the cerebellar cortex correlated well with the individual plasma 14C/glucose ratios, irrespective of drug treatment (Fig 4.2.B-4.3.B). This data indicated that Org 26576 and Org 24448 treatment did not alter glucose utilisation significantly in the cerebellar cortex, authenticating its use as the control reference region.
### A

**Org 26576**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>0.1 mg/kg</th>
<th>1.0 mg/kg</th>
<th>10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Plasma Glucose (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.1 ± 1</td>
<td>11.2 ± 1.3</td>
<td>11.3 ± 0.6</td>
</tr>
<tr>
<td>Terminal Plasma $^{14}$C (nCi/g)</td>
<td></td>
<td>56.8 ± 9.8</td>
<td>60.8 ± 13</td>
<td>61.5 ± 16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>65.0 ± 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

**Org 24448**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>3 mg/kg</th>
<th>10 mg/kg</th>
<th>30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Plasma Glucose (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.2 ± 1.2</td>
<td>10.9 ± 1.4</td>
<td>10.4 ± 2.2</td>
</tr>
<tr>
<td>Terminal Plasma $^{14}$C (nCi/g)</td>
<td></td>
<td>57 ± 13.8</td>
<td>59 ± 11.9</td>
<td>70 ± 5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>67 ± 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Fig 4.1.** Baseline physiological parameters for (A) Org 26576 (0.1, 1, 10 mg/kg) and (B) Org 24448 (3, 10, 30 mg/kg) treated C57Bl/6J mice and their respective vehicle treated groups

Terminal plasma glucose concentrations (mM) and terminal plasma $^{14}$C concentrations (nCi/g) for Org 26576 (A) and Org 24448 (B) treated mice are shown. There were no significant differences between all drug treatments in comparison to their relevant vehicle groups, for either terminal plasma glucose or $^{14}$C levels. Data are presented as mean ± S.D (One-way ANOVA with Dunnett’s correction for multiple comparisons).
Fig 4.2. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for vehicle (Mulgofen-Saline) and Org 26576 (0.1, 1, 10 mg/kg) treated C57Bl/6J mice

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The 'control' relationship (B) was similar between vehicle, and all doses of Org 26576. Data are expressed as mean ± SEM (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Fig 4.3. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for vehicle (Mulgofen-Saline) and Org 24448 (3, 10, 30 mg/kg) treated C57Bl/6J mice

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The 'control' relationship (B) was similar between vehicle, and all doses of Org 24448. Data are expressed as mean ± SEM (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
4.3.1.2. Effects of Org 26576 and Org 24448 on LCGU

Alterations in LCGU following treatment with Org 26576 (0.1, 1, 10 mg/kg) and Org 24448 (3, 10, 30 mg/kg) are presented in Tables 4.1., 4.2. & 4.3.. Both Ampakines induced marked dose-dependent increases in LCGU, with no reductions in LCGU observed in any of the 43 regions analysed after either drug treatment at all doses.

Org 26576 elicited a dose-dependent increase in LCGU in specific brain regions. In all 43 regions examined, there were no significant alterations in LCGU in mice treated with Org 26576 at 0.1 mg/kg as compared to vehicle treated animals. However, treatment with Org 26576 (1 mg/kg) produced significant increases in LCGU in 9 of the 43 anatomical areas examined, including the dentate gyrus, CA3, and several regions of the limbic system (Fig 4.4.). Org 26576 (10 mg/kg) produced significant increases in glucose use in 39 of the 43 anatomical areas examined. Increases were widespread but prominent in the auditory, visual, hippocampal and limbic systems.

Similarly Org 24448 demonstrated a dose-dependent increase in LCGU in specific brain regions. Org 24448 (3 mg/kg) elicited changes in LCGU in only 4 specific areas of the 43 measured, specifically; the raphe nucleus, the lateral habenula nucleus (Fig 4.4.), the median forebrain bundle, and the CA1 field of the hippocampus. Org 24448 (10 mg/kg) produced further significant increases in LCGU, with 31 out of the 43 discrete anatomical areas now reaching significance. Again, notable changes were witnessed in the auditory, visual, hippocampal and limbic systems. Administration of Org 24448 (30 mg/kg) elicited significant changes in LCGU in 34 of the 43 anatomical areas measured. This response (30 mg/kg) was very similar to the response seen with 10 mg/kg, with further activation in certain specific areas such as the anteroventral thalamus, substantia nigra pars compacta and reticulata, and parts of the subicular complex. LCGU in other areas such as the amygdala, caudate, fornix, and globus pallidus was unaffected at all administered doses of Org 24448.
### Table 4.1. Effects of Org 26576 and Org 24448 on LCGU in key components of the limbic system

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D.  
*P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Table 4.2. Effects of Org 26576 and Org 24448 on LCGU in the primary auditory and visual systems

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D.  
*P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
### Ratio of tissue $^{14}$C (nCi/g) in regions of interest to that in the cerebellar cortex

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Org 26576</th>
<th></th>
<th></th>
<th></th>
<th>Org 24448</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>0.1 mg/kg (n=7)</td>
<td>1 mg/kg (n=5)</td>
<td>10 mg/kg (n=6)</td>
<td>Vehicle</td>
<td>3 mg/kg (n=5)</td>
<td>10 mg/kg (n=6)</td>
<td>30 mg/kg (n=6)</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subiculum</td>
<td>1.13 ± 0.07</td>
<td>1.14 ± 0.07</td>
<td>1.23 ± 0.05*</td>
<td>1.31 ± 0.05**</td>
<td>1.16 ± 0.05</td>
<td>1.16 ± 0.02</td>
<td>1.21 ± 0.03</td>
<td>1.21 ± 0.03</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>0.99 ± 0.05</td>
<td>1.09 ± 0.07</td>
<td>1.10 ± 0.08</td>
<td>1.21 ± 0.03**</td>
<td>1.00 ± 0.05</td>
<td>1.04 ± 0.03</td>
<td>1.12 ± 0.07**</td>
<td>1.14 ± 0.03**</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>1.47 ± 0.08</td>
<td>1.52 ± 0.07</td>
<td>1.54 ± 0.07</td>
<td>1.66 ± 0.08**</td>
<td>1.49 ± 0.03</td>
<td>1.51 ± 0.03</td>
<td>1.54 ± 0.03</td>
<td>1.62 ± 0.04**</td>
</tr>
<tr>
<td>Hippocampus - str.mol.</td>
<td>1.37 ± 0.04</td>
<td>1.40 ± 0.03</td>
<td>1.39 ± 0.02</td>
<td>1.56 ± 0.03**</td>
<td>1.38 ± 0.04</td>
<td>1.41 ± 0.04</td>
<td>1.55 ± 0.05**</td>
<td>1.52 ± 0.05**</td>
</tr>
<tr>
<td>Dentate gyrus - str.mol.</td>
<td>0.71 ± 0.03</td>
<td>0.74 ± 0.04</td>
<td>0.76 ± 0.03</td>
<td>0.82 ± 0.04**</td>
<td>0.72 ± 0.02</td>
<td>0.73 ± 0.02</td>
<td>0.92 ± 0.02**</td>
<td>0.94 ± 0.03**</td>
</tr>
<tr>
<td>Medial septum</td>
<td>1.16 ± 0.07</td>
<td>1.13 ± 0.10</td>
<td>1.24 ± 0.08</td>
<td>1.28 ± 0.08*</td>
<td>1.12 ± 0.07</td>
<td>1.13 ± 0.16</td>
<td>1.19 ± 0.05</td>
<td>1.17 ± 0.05</td>
</tr>
<tr>
<td>Dentate gyrus (dorsal)</td>
<td>1.05 ± 0.03</td>
<td>1.05 ± 0.03</td>
<td>1.16 ± 0.04**</td>
<td>1.17 ± 0.07**</td>
<td>1.07 ± 0.03</td>
<td>1.06 ± 0.02</td>
<td>1.23 ± 0.03**</td>
<td>1.21 ± 0.02**</td>
</tr>
<tr>
<td>Hippocampus - CA1</td>
<td>0.81 ± 0.03</td>
<td>0.84 ± 0.04</td>
<td>0.84 ± 0.05</td>
<td>0.97 ± 0.03**</td>
<td>0.78 ± 0.04</td>
<td>0.90 ± 0.05**</td>
<td>0.97 ± 0.03**</td>
<td>0.99 ± 0.04**</td>
</tr>
<tr>
<td>Hippocampus - CA3</td>
<td>0.96 ± 0.06</td>
<td>0.96 ± 0.04</td>
<td>1.13 ± 0.03**</td>
<td>1.13 ± 0.04**</td>
<td>1.02 ± 0.03</td>
<td>1.05 ± 0.02</td>
<td>1.15 ± 0.02**</td>
<td>1.13 ± 0.05**</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>0.99 ± 0.05</td>
<td>0.99 ± 0.05</td>
<td>1.03 ± 0.03</td>
<td>1.12 ± 0.04**</td>
<td>1.00 ± 0.02</td>
<td>1.02 ± 0.03</td>
<td>1.06 ± 0.05*</td>
<td>1.07 ± 0.04*</td>
</tr>
<tr>
<td><strong>Functionally Distinct</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red nucleus</td>
<td>1.50 ± 0.06</td>
<td>1.45 ± 0.03</td>
<td>1.45 ± 0.05</td>
<td>1.59 ± 0.06*</td>
<td>1.46 ± 0.05</td>
<td>1.47 ± 0.05</td>
<td>1.54 ± 0.03</td>
<td>1.61 ± 0.07**</td>
</tr>
<tr>
<td>Inferior olivary body</td>
<td>1.45 ± 0.05</td>
<td>1.43 ± 0.07</td>
<td>1.51 ± 0.07</td>
<td>1.70 ± 0.06**</td>
<td>1.44 ± 0.03</td>
<td>1.49 ± 0.04</td>
<td>1.66 ± 0.02**</td>
<td>1.74 ± 0.06**</td>
</tr>
<tr>
<td>Somatosensory cortex (IV)</td>
<td>1.96 ± 0.10</td>
<td>1.93 ± 0.11</td>
<td>2.04 ± 0.05</td>
<td>1.98 ± 0.06</td>
<td>2.05 ± 0.06</td>
<td>2.00 ± 0.07</td>
<td>2.03 ± 0.03</td>
<td>2.03 ± 0.07</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>0.64 ± 0.03</td>
<td>0.68 ± 0.03</td>
<td>0.67 ± 0.03</td>
<td>0.81 ± 0.04**</td>
<td>0.66 ± 0.03</td>
<td>0.67 ± 0.03</td>
<td>0.77 ± 0.02**</td>
<td>0.74 ± 0.04**</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Cerebellar nucleus</td>
<td>1.87 ± 0.06</td>
<td>1.83 ± 0.03</td>
<td>1.90 ± 0.05</td>
<td>2.22 ± 0.07**</td>
<td>1.91 ± 0.08</td>
<td>1.95 ± 0.09</td>
<td>2.08 ± 0.07*</td>
<td>2.17 ± 0.10**</td>
</tr>
</tbody>
</table>

Table 4.3. Effects of Org 26576 and Org 24448 on LCGU in the hippocampus (+ associated areas) and functionally distinct brain regions

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
Fig 4.4. Representative $[^{14}C]$-2-deoxyglucose autoradiograms illustrating changes in LCGU in response to Org Ampakines and NBQX

Org 26576 (1 mg/kg) produces increases in LCGU in the laterodorsal thalamus (A)ii compared with vehicle administration (A)i. Other notable increases in LCGU are observable in the dentate gyrus (DG), and the CA3 field of the hippocampus. Org 24448 (3 mg/kg) results in an increase in LCGU in the medial lateral habenula (B)ii compared to vehicle administration (B)i. NBQX (10mg/kg) administration alone results in decreased LCGU in the auditory cortex (IV) (C)ii when compared to vehicle administration (C)i.
4.3.2. AMPA receptor antagonist pre-treated mice

NBQX, an AMPA receptor antagonist, was employed to determine if the effects on LCGU by Org 26576 and Org 24448 were directly AMPA receptor mediated. NBQX was administered at 10 mg/kg, while Org 26576 and Org 24448 were also both administered at 10mg/kg, as these doses elicited a robust response in LCGU (see previous). Previous studies indicated that at 10 mg/kg, NBQX was pharmacologically effective with minimal significant effects on glucose use (Brown and McCulloch, 1994), and confirmed that the timings utilised in this study were ample to allow effective blood-brain-barrier penetration and AMPA receptor blockade.

4.3.2.1. Effects of the NBQX on behavioural and baseline physiological parameters

Mice receiving Vehicle-Vehicle, NBQX-Org 26576 or NBQX-Org 24448 did not exhibit any overt or abnormal changes in their behaviour throughout the duration of the experiment. However, mice receiving NBQX followed by vehicle, after the initial injection displayed a lethargy that was evident for the remaining duration of the experiment. Even though these mice were quiet and sedentary, they were cognisant and responsive to stimuli.

The mean terminal plasma $^{14}$C and glucose concentrations in NBQX-vehicle, NBQX-Org 26576 and NBQX-Org 24448 treated mice did not differ significantly from mean terminal plasma $^{14}$C and glucose concentrations for the vehicle-vehicle treated group (Table 4.4.). Mean $^{14}$C levels in cerebellar cortex did not differ significantly in the four treatment groups. In individual animals $^{14}$C levels in the cerebellar cortex correlated well with individual plasma $^{14}$C/glucose ratios, irrespective of drug treatment (Fig 4.5.).

4.3.2.2. Effects of NBQX pre-treatment on LCGU

The effects of pre-treatment with the AMPA receptor antagonist NBQX on LCGU in freely moving conscious mice are presented in Table 4.5. Mice treated with NBQX (10 mg/kg) then vehicle produced significant alterations in LCGU in only 5 of the 43 anatomical areas examined as compared to vehicle-vehicle treated mice. Decreases
<table>
<thead>
<tr>
<th></th>
<th>NBQX Vehicle</th>
<th>NBQX (10 mg/kg) Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug Vehicle (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug Vehicle (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Org 26576 (10 mg/kg) (n = 6)</td>
<td>11.5 ± 1.3</td>
<td>11.7 ± 0.9</td>
</tr>
<tr>
<td>Org 24448 (10 mg/kg) (n = 6)</td>
<td>11.7 ± 0.9</td>
<td>11.7 ± 0.9</td>
</tr>
</tbody>
</table>

Table 4.4. Baseline physiological parameters for Vehicle-Vehicle (Mulgofen-Saline), NBQX-Vehicle, NBQX-Org 26576 and NBQX-Org 24448 treated C57Bl/6J mice

Terminal plasma glucose concentrations (mM) and terminal plasma $^{14}$C concentrations (nCi/g) for Vehicle-Vehicle, NBQX-Vehicle, NBQX-Org 26576 and NBQX-Org 24448 treated mice are shown. There were no significant differences between all treatment combinations when compared, for either terminal plasma glucose or $^{14}$C levels. Data are presented as mean ± S.D (One-way ANOVA with Dunnett's correction for multiple comparisons).
Fig 4.5. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for Vehicle-Vehicle (Mulgofen-Saline), NBQX-Vehicle, NBQX-Org 26576 and NBQX-Org 24448 treated C57Bl/6J mice.

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The 'control' relationship (B) was similar between all treatment combinations. Data are expressed as mean ± SEM (One-way ANOVA, with Dunnett's post-hoc analysis for multiple comparisons).
in LCGU were prominent in the auditory cortex (IV), visual cortex (IV), laterodorsal thalamus, presubiculum and subiculum.

The marked alterations in LCGU in a number of brain regions produced with Org 26576 and Org 24448 were markedly ameliorated by pre-treatment with NBQX (Fig 4.6.). In mice pre-treated with NBQX (10 mg/kg) then Org 26576 (10 mg/kg) only 4 changes in LCGU were found when compared with NBQX (10 mg/kg) alone treated mice. Increases in LCGU were evident in the auditory and visual cortices (IV) and in the inferior olivary body. One significant decrease in LCGU was observed in the cochlear nucleus.

Similarly, mice pre-treated with NBQX (10 mg/kg) then Org 24448 (10 mg/kg) displayed only 3 significant changes in LCGU as compared to NBQX alone treated mice. Increases were evident in the auditory cortex (IV) and the interpenduncular nucleus, while one decrease was observed in the substantia nigra pars compacta region.
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Vehicle + Vehicle (n=5)</th>
<th>NBQX + Vehicle (n=5)</th>
<th>NBQX + Org 26576 (n=6)</th>
<th>NBQX + Org 24448 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anteroventral Thalamus</td>
<td>2.00 ± 0.09</td>
<td>1.92 ± 0.02</td>
<td>1.91 ± 0.08</td>
<td>α</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>1.47 ± 0.07</td>
<td>1.42 ± 0.02</td>
<td>1.43 ± 0.06</td>
<td>α</td>
</tr>
<tr>
<td>Interpenduncular nucleus</td>
<td>1.75 ± 0.06</td>
<td>1.79 ± 0.03</td>
<td>1.76 ± 0.04</td>
<td>α</td>
</tr>
<tr>
<td>Laterodorsal thalamus</td>
<td>1.61 ± 0.05</td>
<td>1.48 ± 0.08</td>
<td>1.55 ± 0.08</td>
<td>α</td>
</tr>
<tr>
<td>Medial Lateral Habenula</td>
<td>1.63 ± 0.05</td>
<td>1.60 ± 0.06</td>
<td>1.60 ± 0.05</td>
<td>α</td>
</tr>
<tr>
<td>Raphe Nucleus</td>
<td>1.30 ± 0.03</td>
<td>1.30 ± 0.03</td>
<td>1.32 ± 0.06</td>
<td>α</td>
</tr>
<tr>
<td>SN pars compacta</td>
<td>1.34 ± 0.04</td>
<td>1.33 ± 0.02</td>
<td>1.29 ± 0.07</td>
<td>α</td>
</tr>
<tr>
<td>Auditory cortex (IV)</td>
<td>1.85 ± 0.09</td>
<td>1.68 ± 0.05          *</td>
<td>1.83 ± 0.03</td>
<td>α β</td>
</tr>
<tr>
<td>Cochlear nucleus</td>
<td>2.00 ± 0.05</td>
<td>2.05 ± 0.09</td>
<td>1.95 ± 0.05</td>
<td>α</td>
</tr>
<tr>
<td>Visual cortex (IV)</td>
<td>1.75 ± 0.02</td>
<td>1.59 ± 0.01          ***</td>
<td>1.71 ± 0.04</td>
<td>α β</td>
</tr>
<tr>
<td>Subiculum</td>
<td>1.21 ± 0.03</td>
<td>1.14 ± 0.02          *</td>
<td>1.18 ± 0.02</td>
<td>α</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>1.02 ± 0.01</td>
<td>1.01 ± 0.03</td>
<td>0.99 ± 0.02</td>
<td>α</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>1.53 ± 0.02</td>
<td>1.48 ± 0.03</td>
<td>1.44 ± 0.03</td>
<td>α</td>
</tr>
<tr>
<td>Hippocampus - str. mol.</td>
<td>1.36 ± 0.01</td>
<td>1.38 ± 0.02</td>
<td>1.38 ± 0.02</td>
<td>α</td>
</tr>
<tr>
<td>Dentate gyrus - str. mol.</td>
<td>0.70 ± 0.00</td>
<td>0.70 ± 0.03</td>
<td>1.71 ± 0.02</td>
<td>α</td>
</tr>
<tr>
<td>Inferior olivary body</td>
<td>1.46 ± 0.04</td>
<td>1.44 ± 0.03</td>
<td>1.25 ± 0.05</td>
<td>α</td>
</tr>
</tbody>
</table>

Table 4.5. Effects of the AMPA receptor antagonist NBQX on LCGUαβ alone, and administered prior to Org 26576 (10 mg/kg) and Org 24448 (10 mg/kg)

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. One-way ANOVA, followed by post-hoc student's t-test with Bonferroni correction for multiple comparisons between groups (correction factor of 5). ***P<0.001, **P<0.01, *P<0.05, for comparison between NBQX pre-treatment and vehicle treated mice. ++P<0.01, +P<0.05, for the comparison between NBQX-26576\(\alpha\)/24448\(\beta\) treatment group with their respective Org 26756 (10 mg/kg) and Org 24448 (10 mg/kg) alone treatment groups.
Fig 4.6. Effects of NBQX (10 mg/kg) administration prior to Org (10 mg/kg) or Org 24448 (10 mg/kg) on LCGU in C57Bl/6J mice

NBQX, an AMPA receptor antagonist, ameliorates the effects of both Org 26576 (A) and Org 24448 (B) on LCGU in the CA3 and CA1 sub-fields of the hippocampus respectively. Data are presented as mean ± SEM. **P<0.01 (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
4.4. Discussion

The aim of this study was to identify the discrete anatomical circuitry influenced by the two novel Ampakines Org 26576 and Org 24448 to investigate their potential therapeutic relevance. The results indicate that both Org 26576 and Org 24448 produce regionally selective, dose-dependent, AMPA receptor mediated increases in LCGU in the mouse cerebrum, with effects indicative of potential therapeutic application in the treatment depression, schizophrenia (psychoses), and associated cognitive deficits.

4.4.1. Org 26576 and Org 24448 produce specific effects on LCGU

The present study demonstrates that Org 26576 and Org 24448 exhibit a clear dose-dependent effect, with specific actions on glucose utilisation at low doses and increasing cerebrum wide activation at higher doses. Further to this Org 26576 and Org 24448 both demonstrate differing patterns of anatomical activation at low doses. Whereas Org 24448 (3 mg/kg) resulted in increased glucose use in the raphe nucleus, medial lateral habenua, CA1 and median forebrain bundle, Org 26576 (1 mg/kg) treatment brought about activation of the DG, CA3, subiculum, anterodorsal and laterodorsal thalamus, the median forebrain bundle, cingulate and visual (IV) cortices and the cochlear nucleus (minor sensory activation). One report in the literature utilising fully-quantitative $[^{13}C]$-2-deoxyglucose autoradiography in the rat, with the structurally distinct biarylpropylsulfonamide AMPA receptor potentiator, LY404187, displayed broadly similar changes in LCGU at a similar dose range to those described in this study (Fowler et al., 2004). Interestingly, no significant effect on LCGU by LY404187 was observed at the higher dose of test compound, suggesting a narrow dose range to elicit alterations in LCGU. In contrast, this bell-shaped dose response was not observed with either Org 26576 or Org 24448. A further study examined the effects of LY404187 in anesthetised rats on BOLD levels in an fMRI study, and discovered that LY404187 produced increases in BOLD most notably in the habenula, hippocampus and the colliculus regions (Jones et al., 2005). A recent study also demonstrated differential effects of two Ampakines (CX546 and CX516) in specific brain regions (hippocampus and thalamus) on electrophysiological synaptic responses (Xia et al., 2004). This was suggested to be due to regional
differences in GluR subunits. The differential responses in LCGU between Org 26576 and Org 24448 at low dose in this study may be due to a similar differential action on specific GluR subunits. This specificity may be lost however with higher doses of the Org compounds, resulting in the robust global activation of AMPA receptors, and hence the similar cerebrum-wide activation observed by both Org 26576 and Org 24448.

4.4.2. AMPA receptor distribution

Extensive autoradiographic binding studies with $[^3]$H-AMPA (Rainbow et al., 1984; Monaghan et al., 1984) and in situ analysis for AMPA receptor subunits (van den Pol et al., 1994; Young et al., 1995; Tomiyama, 1997) in the rat and human brain have shown that AMPA receptor isoforms are distributed heterogeneously across the cerebrum, with high levels of expression in areas such as the hippocampus (especially in the CA1 and CA3 sub-fields), the subiculum, septum, striatum and in superficial cortical layers. The thalamus and hypothalamus display low uniform levels of expression, whilst areas such as the habenula and the dorsal raphe nucleus exhibit moderate levels of AMPA receptor expression (for specifics see section 1.2.6.). Examined in the context of the present study Org 24448 (3mg/kg) therefore produces activation in areas that both have high levels of AMPA receptors, e.g. the CA1 field of the hippocampus, as well as areas with moderate-low levels of expression such as the habenula/raphe. Similarly Org 26575 (1mg/kg) exhibits activation in areas with high AMPA receptor densities, CA3 and cortical areas, but also exhibits activation in the thalamus, which has low levels of expression. Hence the activation of the various brain regions with Org 24448 and Org 26576 does not correspond fully to areas know to classically exhibit AMPA receptors. It is likely therefore that the magnitude of changes observed within different brain regions by the Ampakines Org 24448 and Org 26576 may be due to secondary circuitry (polysynaptic) activation.
4.4.3. The effects of Org 26576 and Org 24448 on LCGU are AMPA receptor mediated

The above-described pattern of glucose use induced with these novel Ampakines is distinctly unlike the patterns of activation seen with other drugs that impinge upon normal glutamatergic transmission such as the NMDA receptor antagonist MK-801 (Kelly et al., 2002) or metabotropic glutamate receptor agonists (Kurumanji et al., 1989). It is also dissimilar to studies in which AMPA stimulation is amplified, (Browne et al., 1998) through intra-cerebral AMPA infusion, but is broadly similar to the cerebral activation induced by the LY biarylsulphonamide AMPA receptor potentiators (Fowler et al., 2004; Jones et al., 2005). Further to this, NBQX, a specific AMPA receptor antagonist with 30-50 fold higher selectivity for AMPA over kainate receptors has been investigated comprehensively in previous [14C]-2-deoxyglucose imaging studies (Brown and McCulloch, 1994). In the rat model, NBQX produces reductions (no increases) in LCGU in the primary auditory areas, various limbic structures and the thalamic nuclei. These previously described effects are comparable to that observed in this study with 10mg/kg i.p. NBQX treatment of mice. Here NBQX produced significant reductions in LCGU in only 5 of the 43 areas examined, specifically the visual and auditory cortices, the laterodorsal thalamus and parts of the subicular complex. NBQX pre-administered prior to Org 24448 and Org 26576 (10 mg/kg) blocked the effects of these novel Ampakines on LCGU confirming that the effects on LCGU observed with Org 24448 and Org 26576 are indeed directly AMPA receptor mediated changes. It should also be noted that NBQX was administered at a much lower dose than that previously reported to induce hypothermia. A previous study (Nurse & Corbett, 1996) indicated a 90 mg/kg dose of NBQX would induce hypothermia in the order of 1-1.5 °C approximately 2hrs post administration. However in this study, the combination of low dose and shortened time course ensured that any possible drug-induced hypothermia would have no discernable effect on the experimental outcome.

4.4.4. Functional implications

The glutamatergic system is possibly the best classified/studied of all the excitatory amino acid systems in the brain, and as a consequence of this, there is now a large
body of evidence implicating glutamate in the pathophysiology of various neurodegenerative diseases and conditions such as cognition, learning and memory impairment, depression and neuropsychoses such as schizophrenia.

With the first true description of AMPA receptor potentiators in the literature (Ito et al., 1990) attention initially focused on the role of these drugs specifically as ‘cognitive enhancers’. The hippocampus and various associated cortical regions have been identified as key areas in the formation of memory (Manns et al., 2003). The Ampakines utilised in this study, Org 26576 and Org 24448 both produce activation of the hippocampus (CA3 and CA1 respectively), and various cortical areas including the cingulate and visual cortices, suggesting that these compounds have the ability to activate brain areas involved in memory formation and storage. This data taken in conjunction with the proven ability of Ampakines to augment synaptic transmission through the facilitation of long-term potentiation (LTP) (Staubli et al., 1994 a/b), currently the leading candidate for memory storage (Lisman, 2001), may provide a role for these compounds in the treatment of mild cognitive impairments (MCI) associated with various conditions such as Alzheimer’s disease, depression, psychoses and ADHD.

Depression is commonly associated with cognitive dysfunction that can be observed as impaired learning and memory (Knapp et al., 2002). As a result of this there is growing evidence that Ampakines may be of therapeutic usefulness in treating depression (Ge et al., 2001; Li et al., 2001; Knapp et al., 2002). The brain circuitry involved in the modulation of depression is well characterised through human brain imaging studies; major areas involved include the locus coreuleus and the raphe nucleus, the origins of the noradrenergic and serotonergic afferents in the brain respectively, the habenula, hippocampus and prefrontal cortical areas (Paul and Skolnick, 2004). Results in this study with Org 24448 (3 mg/kg) demonstrate activation of a number of these areas including the raphe nucleus, lateral habenula, and the hippocampus, suggesting that this compound activates areas associated with depression circuitry in the brain. Although data is unavailable for the locus coreuleus, limited by the power of resolution provided by $[^{14}\text{C}]-2$-deoxyglucose
imaging in the mouse, work by Fowler et al. (2004), in rats, identified an increase in LCGU in this discrete region after acute treatment with the AMPA receptor potentiator LY404187. Moreover, Ampakines have been demonstrated to modulate serotonin within the frontal cortex, mediated through a 5-HT₁₆ mechanism (Ge et al., 2001). Furthermore it has been demonstrated that both in vivo and in vitro positive modulation of the AMPA receptor increases BDNF gene expression in neurones (Legutko et al., 2001; Mackowiak et al., 2002) and neurogenesis in a dose-dependant manner (Bai et al., 2003), while Ampakines such as CX516, CX546 and CX614, have the ability to modulate the in vivo and in vitro levels of BDNF expression in the hippocampus (Lauterborn et al., 2000; Lauterborn et al., 2003), most likely via the Lyn-mediated-MAPK activation pathway (Hayashi et al., 1999). This lends further support to the use of such compounds as possibly efficacious in the treatment of depression as neurotrophins such as BDNF have the ability to modify synaptic transmission and connectivity (plasticity) (Schinder and Poo, 2000) and are major mediators of anti-depressant effects (Santarelli et al., 2003; Duman, 2004; Hashimoto et al., 2004). Depression is also thought to be associated with compromised cell turnover in the dentate gyrus of the hippocampus.

Schizophrenia is associated with neuronal dysregulation and there is now an extensive body of evidence implicating glutamatergic abnormalities in this disorder (Tamminga et al., 2000). Moreover, hypoactive glutamatergic influence in key brain regions, as a result of abnormal expression of all three families of ionotropic glutamate receptors, have been proposed to underlie the psychiatric disturbances witnessed in schizophrenia and related disorders (Hess et al., 2003). Indeed, PET scanning (Tamminga et al., 2000) and expression studies (Meador-Woodruff et al., 2001) in schizophrenic patients have identified key brain areas with compromised glutamatergic transmission, including limbic circuitry such as the striatum, thalamus and hippocampus, and associated efferent pathways and target areas such as the cingulate cortex. Previous studies are suggestive that Ampakines may be useful in effecting depressed cerebral activity associated with schizophrenia (Palmer et al., 1997; Hess et al., 2003). The selective anatomical activation patterns observed in the mouse brain in this study, lends further support to this concept. Treatment with both
Org 26576 and Org 24448, especially Org 26576 (1mg/kg), elicited specific increases in glucose use in brain regions associated with schizophrenia such as the hippocampus, cingulate cortex and various thalamic regions. Ampakines therefore may potentially facilitate glutamatergic transmission/activation in these ‘deficient’ areas and subsequently counter the neurotransmitter imbalance to alleviate the symptoms of schizophrenia. Previous studies investigating the acute effects of psychoactive compounds in rat brain have demonstrated alterations in LCGU in particular brain regions characteristic of the compound class. For example, following acute administration of the antipsychotic compounds haloperidol, clozapine, olanzapine and the novel antipsychotic compound asensapine, an increase in LCGU within the lateral habenula is observed (McCulloch et al., 1980; Room et al., 1991; Duncan et al., 2003). This increase in glucose use within the lateral habenula was also observed in this study following acute administration of Org 26576 or Org 24448. However, in contrast, these antipsychotic compounds also reduced levels of LCGU within the thalamus, an effect not observed following acute administration of the Ampakines used in this study. It is likely that these differences reflect the different pharmacological actions of the compounds and subsequent effects on extended neuronal circuitry.

4.4.5. Summary

The results of the present study demonstrate that both Org 26576 and Org 24448 produce dose-dependent AMPA receptor mediated changes in LCGU in the mouse cerebrum with specific but distinct regional activation. In conclusion, these data provide an anatomical basis for the cerebral activation induced by these compounds, and are suggestive of a potential therapeutic benefit for conditions implicated with an associated glutamatergic hypofunction, such as depression and schizophrenia, or cognitive deficits.
Chapter 5

Investigation into the effects of *chronic* administration of the novel Ampakines Org 26576 and Org 24448 on functional activity, neurogenesis and receptor/signalling alterations in the murine cerebrum
5.1. Introduction

Current treatments for conditions such as depression and schizophrenia, whilst effective in treating and controlling the symptoms of these diseases are not without their limitations. Major drawbacks can include prolonged times of onset of therapeutic effects, 6-8 weeks for example with most anti-depressants (Tamminga et al., 2002), dramatic side effects, such as tardive dyskinesia with anti-psychotics, and overall treatment efficacy can be poor with relapses commonplace (Nestler et al., 2002; Zajecka, 2003). Having proven the ability of the novel Org Ampakines to modulate central cerebral function in areas associated with cognition, depression and psychoses in an acute paradigm (Chapter 3) it is essential to investigate their more long-term ‘chronic’ functional effects to establish if any changes witnessed are similar both in magnitude and locality in comparison to the acute effects; and whether or not the onset of these effects are rapid, but more importantly maintained over time.

In addition to overall functional changes it is imperative to try and understand the underlying ‘structural’ correlates to these functional changes, and attempt to dissect the down-stream consequences of cerebral activation. Indeed, AMPA receptor-potentiating compounds when administered systemically have been shown to increase levels of brain-derived neurotrophic factor (BDNF) (Legutko et al., 2001; Mackowiak et al., 2002), activate ERK/CREB second messenger systems (Hayashi et al., 1999), and engender neurogenesis in vivo (Bai et al., 2003). These ‘structural’ effects are produced by traditional antidepressants, and are often implicated and linked to their antidepressant properties (Warner-Schmidt & Duman, 2006). It is still a matter of conjecture however if Ampakines produce their effects through a specific delineated structural pathway or via a potential novel mechanism.

5.1.1. Aims

The aim of the current study was to investigate the effects of chronic administration of the novel Ampakines Org 26576 and Org 24448 in the mouse cerebrum. Functional activity was assayed using semi-quantitative [14C]-2-deoxyglucose autoradiography. ‘Structural alterations’ were investigated, specifically in the
hippocampus, by examining the ability of chronic Ampakine administration to modify the number of proliferating dentate gyrus cells, and Western blot analysis, to investigate the effects of chronic administration on total AMPA GluR levels and down-stream signalling components implicated in AMPA/Ampakine function.

5.2. Materials and Methods

5.2.1. Chronic drug administration

The experimental study design is outlined above. The Ampakines Org 26576 and Org 24448 were made up as stock solutions prior to the beginning of the study, aliquoted, and stored at -20°C until used. Each day the required volume of compound was defrosted and made up to the final appropriate dosage in a 0.2ml bolus per mouse. C57Bl/6J mice were evenly distributed across treatment groups by weight, and were intraperitoneally injected twice daily, 8hrs part, in opposing sides of the abdomen with either vehicle (Mulgofen-saline), Org 26576 (1 mg/kg) or Org 24448 (10 mg/kg). Injections were intermittently checked for correct peritoneal placement, by insertion of needle and drawing back on the syringe. If air was drawn into the syringe the needle was deemed to be correctly placed. Animals in Group 1 received injections of Vehicle, Org 26576 and Org 24448 for 7 full days, whilst...
animals in Group 2 received injections for a full 28 days (n=8 for each treatment and time point). This experimental design was carried out in duplicate to allow investigation of two distinct endpoints: 1) functional investigation using $[^{14}C]2$-deoxyglucose autoradiography, and 2) investigation into structure (neurogenesis) and signalling using immunohistochemistry and Western blot analysis (Fig 5.1.).

5.2.2. $[^{14}C]2$-deoxyglucose autoradiographic procedure

After either 7 or 28 days of vehicle, Org 26576 or Org 24448 treatment, the mice were subjected to $[^{14}C]2$-deoxyglucose autoradiography to examine the functional consequences of chronic administration on cerebral activity. The morning following the final drug injection (a 24 hr ‘washout’ period) mice were separated into individual cages in the procedure room and allowed to acclimatise to their new environment for 30 min. The procedure was carried out the next morning to ensure any effects witnessed were chronic effects and not residual acute drug effects. $5 \mu Ci$ $[^{14}C]2$-deoxyglucose was administered intraperitoneally in a 0.4 ml bolus over a 10sec period. The detailed autoradiographic experimental procedure is outlined in section 2.2.3. Standard autoradiographs were prepared and LCGU was determined in pre-determined areas of interest as described in section 2.2.4. All experiments were controlled as stringently as possible for light and sound conditions, and circadian timings to ensure equal distribution of variables across the treatment groups.

5.2.3. BrdU administration and immunohistochemistry

After either 7 or 28 days of vehicle, Org 26576, or Org 24448 treatment mice were intraperitoneally injected with (+)5-Bromo-2'-deoxyuridine (BrdU) (75 mg/kg), a base analogue of thymidine that incorporates itself into a proliferating cell’s DNA during S phase and can subsequently be detected by immunohistochemistry (Nakagawa et al., 2002). Following the BrdU injection (12 hrs after the last drug injection), mice were allowed to survive for a further 24 hrs to allow full cellular incorporation of the BrdU (at least 3 mammalian cell cycles) after which they were transcardially perfused with 0.9% heparinised saline as described in section 2.3. The brains when removed were initially hemi-transected along their midline and half the brain underwent micro-dissection as outlined in section 2.4. The other half was post-fixed in 4% paraformaldehyde in 50 mM phosphate buffer solution for 24 hrs,
cryoprotected in 30% sucrose in 50 mM phosphate buffer solution for 24hrs, frozen in chilled isopentane (-42 °C) for 5 min and stored in 7 ml bijou tubes at –20 °C for sectioning. 20 μm cryostat sections were prepared and retained on poly-l-lysine coated slides as detailed in Section 2.5. for BrdU-immunohistochemistry. Immunohistochemistry was performed as described in section 2.6.2. using a mouse monoclonal anti-BrdU antibody (1:100 - Sigma Aldrich, UK).

5.2.4. Quantification of BrdU positive nuclei
The number of BrdU-positive cells in three serial sections per animal of the hippocampus at the level of the medial lateral habenula (Bregma: -1.82 mm) were counted in a blinded-randomised manner. A modified version of the optical fractionator method for unbiased stereological analysis of the total number of BrdU-positive cells in the hippocampal subdivision was utilized as previously reported (West et al., 1991). A cell was counted as being in the subgranular zone (SGZ) of the dentate gyrus if it was touching or in the SGZ (Fig 5.2.); cells located 2 or more cells away from the SGZ were classified as hilar. All BrdU-positive cells were counted through a 100x objective light microscope, throughout the rostrocaudal extent of the hippocampal granule cell layer.

![Fig 5.2. BrdU-positive immunostaining in the dentate gyrus](image)

Large darkly stained cluster of BrdU-positive nuclei (arrow) in the subgranular zone of the dentate gyrus (x200), and shown enlarged (inset x400).
5.2.5. Protein analysis
Dissected hippocampi from vehicle, Org 26576 and Org 24448 treated mice for 7 or 28-days were homogenised/purified as described in section 2.7.1. Exact protein concentrations for the homogenates were determined using a BCA assay (section 2.7.2.). For each sample (n=~8) 80 μg protein was separated by SDS-PAGE electrophoresis (section 2.7.3.), and underwent Western blotting (section 2.7.4.). Levels of receptors (i.e. GluR’s) and signalling molecules (i.e. CREB) were determined by immuno-blotting with antigen-specific antibodies as described in section 2.6.2.

5.2.6. Statistical analysis
All data obtained were analysed for statistical significance by one-way analysis of variance, followed by Dunnett’s post-hoc analysis, to correct for multiple comparisons between the drug-treated and control groups.
5.3. Results

5.3.1. Functional consequences of chronic administration of Org 26576 and Org 24448

5.3.1.1. Effect of Org 26576 and Org 24448 administration on behavioural and baseline physiological parameters

Treatment with Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) for 7 and 28 days resulted in no alterations in the overt behaviour of the mice. Mice received injections twice daily and were at all times alert and active displaying normal circadian, exploratory and grooming behaviour. On the day of $^{14}$C-2-deoxyglucose autoradiography mice were separated into individual cages. Immediately following the $^{14}$C-2-deoxyglucose injection mice displayed a period of inactivity during which they remained quietly hunched in the closed portion of the cage. This lassitude lasted only briefly however, approximately ten minutes, after which the mice displayed normal behavioural activity for the remainder of the experimental period.

Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) administration had no significant effect on the mean terminal plasma $^{14}$C and glucose concentrations as compared to the relevant vehicle treated groups for both the 7 and 28 day treatment time points (Fig 5.3.). Mean $^{14}$C levels in the cerebellar cortex did not differ significantly between the vehicle and drug groups, and in individual mice, $^{14}$C levels in the cerebellar cortex correlated well with the individual plasma $^{14}$C/glucose ratios, irrespective of drug treatment or time point (Fig 5.4. & 5.5.). This data is suggestive that chronic injection of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) did not alter glucose utilisation significantly in the cerebellar cortex over prolonged periods of time, verifying its use as the control reference region in this study.
A

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=7)</th>
<th>Org 26576 1 mg/kg (n=7)</th>
<th>Org 24448 10 mg/kg (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Plasma Glucose (mM)</td>
<td>9.87 ± 1.61</td>
<td>9.44 ± 2.07</td>
<td>10.40 ± 1.07</td>
</tr>
<tr>
<td>Terminal Plasma $^{14}$C (nCi/g)</td>
<td>39.33 ± 11.43</td>
<td>43.61 ± 8.12</td>
<td>40.69 ± 5.28</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=7)</th>
<th>Org 26576 1 mg/kg (n=5)</th>
<th>Org 24448 10 mg/kg (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal Plasma Glucose (mM)</td>
<td>10.99 ± 1.32</td>
<td>10.19 ± 1.2</td>
<td>10.2 ± 1.74</td>
</tr>
<tr>
<td>Terminal Plasma $^{14}$C (nCi/g)</td>
<td>44.88 ± 13.02</td>
<td>46.05 ± 8.41</td>
<td>32.73 ± 6.05</td>
</tr>
</tbody>
</table>

Fig 5.3. Baseline physiological parameters for chronically administered Org 26576 (1 mg/kg), Org 24448 (10 mg/kg), and vehicle in C57Bl/6J mice

Terminal plasma glucose concentrations (mM) and terminal plasma $^{14}$C concentrations (nCi/g) for Vehicle, Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) treated mice are shown for 7 days (A) and 28 days (B). There were no significant differences between all drug treatments in comparison to the relevant vehicle group, for either terminal plasma glucose or $^{14}$C levels. Data are presented as mean ± S.D (One-way ANOVA with Dunnett’s correction for multiple comparisons).
Fig 5.4. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C/glucose ratio for vehicle (Mulgofen-Saline), Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg), 7 day treated C57Bl/6J mice.

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The ‘control’ relationship (B) was similar between vehicle, Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg). Data are expressed as mean ± SEM (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
Fig 5.5. Cerebellar cortex $^{14}$C concentration and its relationship with the terminal plasma $^{14}$C / glucose ratio for vehicle (Mulgofen-Saline), Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg), 28 day treated C57Bl/6J mice.

Cerebellar cortex $^{14}$C concentrations (A) for all drug treatments did not differ significantly from vehicle-treated mice. The ‘control’ relationship (B) was similar between vehicle, Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg). Data are expressed as mean ± SEM (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
5.3.1.2 Effects of 7-day administration of Org 26576 (1mg/kg) and Org 24448 (10mg/kg) on LCGU

Alterations in glucose utilisation associated with chronic 7 day administration of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) are presented in Tables 5.1., 5.2., and 5.3. Both Ampakines induced significant marked increases in LCGU across the cerebrum, with no reductions in LCGU observed in any of the 44 regions analysed when compared to the relevant vehicle treated groups.

7-day treatment with Org 26576 (1 mg/kg) produced significant increases in LCGU in 34 of the 44 anatomical areas examined. The greatest increases were witnessed in the hippocampus (stratum molecularae & CA1) and associated structures (subicular complex), the thalamus (anteroventral and laterodorsal components) and its associated target areas (cingulate cortex), the white matter tracts (fornix, MFB) and several key neurotransmitter ‘regions’ such as the raphe nucleus, medial septum, substantia nigra and nucleus accumbens. No significant alterations in LCGU were seen in areas such as the amygdala, hypothalamus, caudate nucleus, entorhinal cortex, corpus callosum and somatosensory cortex.

Similarly, 7-day treatment with Org 24448 (10 mg/kg) produced significant increases in LCGU in 34 of the 44 anatomical areas examined. Prominent increases in LCGU were witnessed primarily in the hippocampus and all its subfields and associated structures (entorhinal cortex, subicular complex), in the thalamus, MFB, medial septum and nucleus accumbens. Other significant increases were observable in the raphe nucleus, cingulate cortex, fornix and substantia nigra. Similarly to Org 26576 no changes were seen in the amygdala, hypothalamus, corpus callosum and somatosensory cortex.

5.3.1.3 Effects of 28-day administration of Org 26576 (1mg/kg) and Org 24448 (10mg/kg) on LCGU

Alterations in LCGU associated with chronic 28 day administration of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) are presented in Tables 5.1., 5.2. and 5.3. Both Ampakines induced significant marked increases in LCGU across the cerebrum, with
### Table 5.1. Effects of chronic administration (7 and 28 days) of the Ampakines Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) on LCGU in key components of the limbic system

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
<table>
<thead>
<tr>
<th>Brain Region</th>
<th>7 Days Administration</th>
<th>28 Days Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Org 26576 (1 mg/kg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg/kg</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>Primary Auditory System</td>
<td></td>
<td>1 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auditory cortex (IV)</td>
<td>1.84 ± 0.03</td>
<td>1.94 ± 0.05**</td>
</tr>
<tr>
<td>Cochlear nucleus</td>
<td>1.87 ± 0.06</td>
<td>2.11 ± 0.13**</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>2.45 ± 0.12</td>
<td>2.53 ± 0.11</td>
</tr>
<tr>
<td>Lateral lemniscus</td>
<td>1.42 ± 0.13</td>
<td>1.62 ± 0.10</td>
</tr>
<tr>
<td>Medial geniculate body</td>
<td>1.53 ± 0.11</td>
<td>1.66 ± 0.10</td>
</tr>
<tr>
<td>Superior olives</td>
<td>1.72 ± 0.09</td>
<td>1.81 ± 0.10</td>
</tr>
<tr>
<td>Primary Visual System</td>
<td></td>
<td>1 mg/kg</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral geniculate body</td>
<td>1.45 ± 0.06</td>
<td>1.70 ± 0.07***</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>1.37 ± 0.05</td>
<td>1.48 ± 0.03**</td>
</tr>
<tr>
<td>Visual cortex (IV)</td>
<td>1.62 ± 0.03</td>
<td>1.83 ± 0.07***</td>
</tr>
</tbody>
</table>

Table 5.2. Effects of chronic administration (7 and 28 days) of the Ampakines Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) on LCGU in the primary auditory and visual systems

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
### Ratio of tissue $^{14}$C (nCi/g) in regions of interest to that in the cerebellar cortex

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>7 Days Administration</th>
<th>28 Days Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>Org 26576</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td><strong>Hippocampus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dentate gyrus (dorsal)</td>
<td>1.02 ± 0.05</td>
<td>1.14 ± 0.05**</td>
</tr>
<tr>
<td>Dentate gyrus – str. mol.</td>
<td>0.72 ± 0.04</td>
<td>0.78 ± 0.02*</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>1.01 ± 0.04</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>Hippocampus – str. mol.</td>
<td>1.34 ± 0.05</td>
<td>1.58 ± 0.05***</td>
</tr>
<tr>
<td>Hippocampus – CA1</td>
<td>0.78 ± 0.05</td>
<td>0.91 ± 0.03***</td>
</tr>
<tr>
<td>Hippocampus – CA3</td>
<td>0.97 ± 0.03</td>
<td>1.07 ± 0.04***</td>
</tr>
<tr>
<td>Medial septum</td>
<td>1.11 ± 0.08</td>
<td>1.26 ± 0.06**</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>1.03 ± 0.05</td>
<td>1.24 ± 0.06***</td>
</tr>
<tr>
<td>Presubiculum</td>
<td>1.54 ± 0.04</td>
<td>1.71 ± 0.08***</td>
</tr>
<tr>
<td>Subiculum</td>
<td>1.19 ± 0.05</td>
<td>1.48 ± 0.06***</td>
</tr>
<tr>
<td><strong>Functionally Distinct</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Cerebellar nucleus</td>
<td>1.88 ± 0.03</td>
<td>1.97 ± 0.08</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>0.66 ± 0.02</td>
<td>0.64 ± 0.02</td>
</tr>
<tr>
<td>Inferior olives</td>
<td>1.41 ± 0.04</td>
<td>1.64 ± 0.03***</td>
</tr>
<tr>
<td>Red nucleus</td>
<td>1.46 ± 0.05</td>
<td>1.58 ± 0.06**</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>2.00 ± 0.06</td>
<td>1.98 ± 0.07</td>
</tr>
</tbody>
</table>

Table 5.3. Effects of chronic administration (7 and 28 days) of the Ampakines Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) on LCGU in the hippocampus (+ associated areas) and functionally distinct brain regions

Data are presented as mean ratio of tissue $^{14}$C (nCi/g) in the regions of interest to tissue $^{14}$C in the cerebellar cortex (nCi/g) ± S.D. *P<0.05, **P<0.01, ***P<0.001, for statistical comparison between vehicle and drug treated groups (One-way ANOVA, with Dunnett’s post-hoc analysis for multiple comparisons).
no reductions in LCGU observed in any of the 44 regions analysed when compared to the relevant vehicle treated groups.

28-day treatment with Org 26576 (1 mg/kg) produced significant increases in LCGU in 31 of the 44 anatomical areas examined. The largest increases were evident in the hippocampus (dentate gyrus, CA1, CA3), and primary components of the mesocorticolimbic system such as the ventral tegmental area, interpeduncular nucleus, mamillary body, fasciculus retroflexus and the median forebrain bundle. Other significant increases were witnessed in the globus pallidus and substantia nigra. No significant alterations in LCGU were seen in areas such as the hypothalamus, corpus callosum, somatosensory cortex and surprisingly the fornix, which displayed significant large increases in LCGU after 7-day administration.

28-day treatment with Org 24448 (1 mg/kg) produced significant increases in LCGU in 35 of the 44 anatomical areas examined. Similarly, 28-day treatment with Org 24448 resulted in a response of similar magnitude to that witnessed with 7-day administration, but with a distinct shift in the profile of areas activated. Areas displaying the greatest activation now included the FR, mamillary bodies, raphe nucleus, superior & inferior olives and the molecular layer of the hippocampus. Other significant increases were evident in the fornix, dentate gyrus, nucleus accumbens, substantia nigra, ventral tegmental area, and interpeduncular nucleus. No changes in LCGU were seen in the caudate nucleus, hypothalamus, corpus callosum and somatosensory cortex.

5.3.2. Structural consequences of chronic administration of Org 26576 and Org 24448

5.3.2.1. BrdU immunostaining and quantification
The number of newly born cells in the adult mouse dentate gyrus was determined by immunohistochemical detection of BrdU within the nuclei of dividing cells. Mice were treated with Org 26576 Org 24448 or vehicle for 7 or 28 days, after which BrdU was injected one day prior to termination. This methodology allows the detection of overall levels of proliferating cells (neurogenesis) to be examined in
response to drug/vehicle administration. Immunostaining was initially optimised, with several pre-treatments, such as trypsin, 1M HCl and citric acid buffer (pH 6.0) applications being trialled to determine optimal staining.

Treatment with both Org 26576 and Org 24448 for 7 and 28 days resulted in no significant changes in the level of BrdU staining in the dentate gyrus when compared to control treatment (Fig 5.6 & 5.7). In 7 day treated mice the average number of BrdU-positive cells in the dentate gyrus of vehicle treated mice was 20.9 ± 1.5 cells, 20.4 ± 0.97 for Org 26576 treated mice, and 22.1 ± 1.89 cells for Org 24448 treated mice. In 28 day treated mice the number of BrdU-positive cells in the dentate gyrus of vehicle treated mice was 15.1 ± 1 cells, 12.77 ± 1.37 cells for Org 26576 treated mice, and 14.6 ± 1.79 cells for Org 24448 treated mice. BrdU-positive nuclei of proliferating cells were localised predominantly in the subgranular zone (border between granule cell layer and hilus) in both chronically Ampakine treated and control mice. Cells frequently appeared as clusters of two or more cells, and were darkly stained with variable shapes and sizes. No differences in cell morphology were evident between chronically treated Ampakine and vehicle treated control mice.

5.3.2.2. Hippocampal protein levels
GluR1 and GluR2/3 were detected as a single protein band of approximately 108KDa. GluR4 was not investigated due to the reported low expression in the hippocampus. In response to both 7 and 28 days treatment with both Ampakines no significant changes in the overall levels of GluR1, 2/3 receptor subtypes were determined as compared to vehicle controls (Fig 5.8 & 5.9).

Downstream intracellular signalling components, all of which have been previously implicated or associated in AMPA and/or Ampakine function were also investigated in the hippocampus of mice treated chronically with both Ampakines (Fig 5.10). CREB, cAMP-response-binding protein, is a transcription factor intimately associated with learning and memory, and ultimately the downstream correlate of BDNF and ERK/Lyn-kinase activation. P-CREB levels were investigated in this
Fig 5.6. BrdU immunostaining in the mouse hippocampus following 7 days administration of vehicle, Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg)

Representative examples of BrdU immunostaining are shown (A) with the quantified data (B). 7 day administration of both Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) resulted in no significant alterations in the level of neurogenesis, investigated by BrdU immunostaining, when compared to chronically treated vehicle mice. Data are expressed as mean ± SEM (One-way ANOVA.) SGZ - subgranular zone; GC - granule cell layer.
Fig 5.7. BrdU immunostaining in the mouse hippocampus following 28 days administration of vehicle, Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg)

Representative examples of BrdU immunostaining are shown (A) with the quantified data (B). 28 day administration of both Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) resulted in no significant alterations in the level of neurogenesis, investigated by BrdU immunostaining, when compared to chronically treated vehicle mice. Data are expressed as mean ± SEM (One-way ANOVA).
study, and no significant differences in protein levels were detected with any chronic application of Ampakines when compared to vehicle controls.

ERK’s, are ~42kDa serine/threonine protein kinases, often termed MAPKinases, which transduce a large variety of extracellular signals leading in turn to a wide range of cellular responses including importantly growth and differentiation, induced by growth factors such as BDNF, which as outlined previously is intimately implicated in Ampakine and antidepressant function. Both native ERK and activated phosphorylated ERK (P-ERK) were detected in the hippocampi after chronic treatment with either vehicle or both Ampakines. No significant differences were detected in the levels of either after 7 and 28-days treatment with Org 26756 or Org 24448.

Lyn-kinase, an Src-family protein tyrosine kinase, which is functionally induced by AMPA receptor activation, and subsequently can in turn induce further BDNF production and ERK activation was also detected in the hippocampus of chronically treated mice. As with ERK, no significant differences in the levels of Lyn-kinase were detected at any time point with any Ampakine treatment. The effects of chronic administration of Org 26576 and Org 24448 were also investigated on levels of calbindin, a 28kDA vitamin D-dependent calcium-binding protein, an indicator of increased levels of Ca\(^{2+}\) influx into cells. Similarly to the other signalling molecules investigated, no significant changes in levels of calbindin were detected between chronic treatments.

Protein loading was assessed by examining the levels of the ‘housekeeping’ protein actin, and by coomassie blue staining of the transferred gels (section 2.7.5.). No significant differences in the levels of actin were detected between the control and Ampakine treated hippocampal samples at either 7 or 28 days. Coomassie blue staining of the gels also confirmed equal loading of the samples.
Fig 5.8. Western blot and quantification of GluR1 protein levels

Levels of GluR1 in the mouse hippocampus are unchanged following 7-day (A) and 28-day (B) administration of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) compared to vehicle controls. Results are shown as both a graph of quantitative data derived from optical density measurements of the western blot, and representative western blots bands for each treatment. Data are expressed as mean ± SEM.
Fig 5.9. Western blot and quantification of GluR2/3 protein levels

Levels of GluR1 in the mouse hippocampus are unchanged following 7-day (A) and 28-day (B) administration of Org 26576 (1 mg/kg) and Org 24448 (10 mg/kg) compared to vehicle controls. Results are shown as both a graph of quantitative data derived from optical density measurements of the western blot, and representative western blots bands for each treatment. Data are expressed as mean ± SEM.
Fig 5.10. Western blots of proteins implicated in AMPA receptor / Ampakine signalling in the murine hippocampus

Representative protein bands are shown for P-CREB, the MAPKinasers (ERK1-2 & phosphorylated forms), P-LynKinase, calbindin and actin (loading control) following either 7 day (A) or 28 day (B) chronic administration of vehicle, Org 26576 (1 mg/kg) or Org 24448 (10 mg/kg). No changes were discernable following treatment with either drug at either time point when compared to the vehicle controls.
5.4. Discussion

The aim of this study was to investigate the effects of chronic administration (7 and 28 days) of the novel Ampakines Org 26576 and Org 24448 in the mouse cerebrum, on distinct functional and structural endpoints. The results are indicative that both Org 26576 and Org 24448 administered chronically induce functional cerebral increases, particularly in areas of the limbic system, which are not only rapid in onset, with significant effects visible after 7 days administration; but importantly are persistent. However, although highly functionally active, chronic administration of these Ampakines had no significant effect on the level of neurogenesis or on the levels of key proteins and signalling pathways implicated in AMPA/Ampakine signalling, in the murine hippocampus.

5.4.1. Effects of chronic administration of Org 26576 and Org 24448 on functional activity in the mouse cerebrum

Both Org 26576 and 24448 following 7 days chronic administration induced increases in LCGU that were foremost evident in key components of the limbic system, especially the hippocampus, and areas associated with the mesocorticolimbic system; including the nucleus accumbens, medial septum and substantia nigra. Both compounds while producing similar overall responses in distinct brain regions displayed a differing overall hierarchy of responses in comparison to each other (Table 5.4. & 5.5.). These results are comparable to the acute effects witnessed in Chapter 4, but with recruitment of distinct new regions. Treatment for 28 days with both compounds resulted in a shift in the profile of activation compared to 7 days with more key limbic (mesocorticolimbic) structures, such as the VTA, interpenduncular nucleus, and mamillary bodies as well as key neurotransmitter areas such as the substantia nigra and raphe nucleus (in the case of Org 24448) displaying pronounced activation. The overall magnitude of response was similar between 7-day and 28-day Org 24448 and Org 26576 treated mice.
<table>
<thead>
<tr>
<th>&gt;20% ↑ in LCGU</th>
<th>&gt;15% ↑ in LCGU</th>
<th>&gt;10% ↑ in LCGU</th>
<th>&gt;5% ↑ in LCGU</th>
<th>&lt;5% ↑ in LCGU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7 Days</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subiculum</td>
<td>AV thalamus</td>
<td>DG (dorsal) / CA3</td>
<td>Medial geniculate body</td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>LD thalamus</td>
<td>FR / VTA</td>
<td>Mammillary body</td>
<td>Cerebellar nucleus</td>
</tr>
<tr>
<td>CA1</td>
<td>Medial septum</td>
<td>SN pars ret. &amp; comp.</td>
<td>Globus pallidus</td>
<td>Inferior colliculus</td>
</tr>
<tr>
<td>Hippocampus (mol.)</td>
<td>Cingulate cortex</td>
<td>Nucleus accumbens</td>
<td>Superior colliculus</td>
<td>Caudate nucleus</td>
</tr>
<tr>
<td>Fornix / MFB</td>
<td>Visual cortex (IV)</td>
<td>Caudate nucleus</td>
<td>MLH</td>
<td>Amygdala</td>
</tr>
<tr>
<td>Raphe nucleus</td>
<td>Lateral lemniscus</td>
<td>Cochlear nucleus</td>
<td>Red nucleus</td>
<td>Cerebellar cortex</td>
</tr>
<tr>
<td>Lateral geniculate body</td>
<td>Interpeduncular nucleus</td>
<td>Interpeduncular nucleus</td>
<td>DG (mol.)</td>
<td>Somatosensory cortex</td>
</tr>
<tr>
<td>Inferior olives</td>
<td>Cerebellar nucleus</td>
<td>Auditory cortex (IV)</td>
<td>Superior olives</td>
<td>Corpus callosum</td>
</tr>
</tbody>
</table>

| **28 Days**    |                |               |               |               |
| DG (dorsal)    | DG (mol) / CA1 / CA3 | Inferior colliculus | LD thalamus | Somatosensory cortex |
| Superior olives| Hippocampus (mol.) | AV thalamus | MFB / FR / MLH | Lateral geniculate body |
| VTA            | SN pars comp.  | SN pars ret. | SN pars ret. | Fornix |
| Interpeduncular nucleus | Globus pallidus | Visual cortex (IV) | Visual cortex (IV) | Corpus callosum |
| nucleus        | Mammillary body | Medial geniculate body | Medial geniculate body | Subiculum |
|                | Caudate nucleus | Presubiculum | Presubiculum | Superior colliculus |
|                | Cingulate cortex | Parahippocampal gyrus | Parahippocampal gyrus | Red nucleus |
|                | Auditory cortex (IV) | Caudate nucleus | Medial septum | Hypothalamus |
|                | Entorhinal cortex | Lateral lemniscus | Lateral lemniscus | Cerebellar cortex |
|                | Inferior olives | Cochlear nucleus | Cochlear nucleus | |
|                | Cerebellar nucleus | Raphe nucleus | Raphe nucleus | |
|                |                 | Amygdala | Amygdala | |
|                |                 | Nucleus accumbens | Nucleus accumbens | |

Table 5.4. Hierarchical comparison of the overall effects on LCGU of Org 26576 (1 mg/kg) administration following 7 and 28 days chronic administration compared to vehicle treated controls.
<table>
<thead>
<tr>
<th></th>
<th>&gt;20% ↑ in LCGU</th>
<th>&gt;15% ↑ in LCGU</th>
<th>&gt;10% ↑ in LCGU</th>
<th>&gt;5% ↑ in LCGU</th>
<th>&lt;5% ↑ in LCGU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>7 Days</strong></td>
<td>Subiculum</td>
<td>AV thalamus</td>
<td>Visual cortex (IV)</td>
<td>Globus pallidus</td>
<td>Amygdala</td>
</tr>
<tr>
<td></td>
<td>Parasubiculum</td>
<td>LD thalamus</td>
<td>Fornix / FR</td>
<td>SN pars ret.</td>
<td>Cerebellar cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA3 / CA1 / DG (all)</td>
<td>Entorhinal cortex</td>
<td>Lateral geniculate body</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hippocampus (mol.)</td>
<td>Raphe nucleus</td>
<td>MLH</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MFB</td>
<td>Superior colliculus</td>
<td>Auditory cortex (IV)</td>
<td>Somatosensory cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medial septum</td>
<td>Cingulate cortex</td>
<td>Mammillary body</td>
<td>Inferior colliculus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nucleus accumbens</td>
<td>Lateral geniculate body</td>
<td>Caudate nucleus</td>
<td>Superior olives</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Presubiculum</td>
<td>VTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cochlear nucleus</td>
<td>Interpeduncular nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inferior olives</td>
<td>Medial geniculate body</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SN pars comp.</td>
<td>Cerebellar nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Red nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>28 Days</strong></td>
<td>FR</td>
<td>Hippocampus (mol.)</td>
<td>Para/Subiculum</td>
<td>CA1</td>
<td>Cerebellar cortex</td>
</tr>
<tr>
<td></td>
<td>Superior olives</td>
<td>Raphe nucleus</td>
<td>DG (all) / CA3</td>
<td>AV thalamus</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mammillary body</td>
<td>LD thalamus</td>
<td>Presubiculum</td>
<td>Hypothalamus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Auditory cortex (IV)</td>
<td>Fornix / MLH</td>
<td>MFB</td>
<td>Somatosensory cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medial geniculate body</td>
<td>VTA</td>
<td>Medial septum</td>
<td>Red nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inferior olives</td>
<td>Interpeduncular nucleus</td>
<td>Globus pallidus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SN pars retc. &amp; comp</td>
<td>Cingulate cortex</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nucleus accumbens</td>
<td>Lateral geniculate body</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Visual cortex (IV)</td>
<td>Caudate nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Entorhinal cortex</td>
<td>Superior colliculus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cochlear nucleus</td>
<td>Amygdala</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inferior colliculus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lateral geniculate body</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.5. Hierarchical comparison of the overall effects on LCGU of Org 24448 (10 mg/kg) administration following 7 and 28-days chronic administration compared to vehicle treated controls.
5.4.2. The mesocorticolimbic system

The major effects on functional activity witnessed with chronic Org administration were localised mainly in the limbic system. Composed primarily of the cingulate gyrus, hippocampus, thalamus (anterior portion) hypothalamus (mamillary bodies), amygdala, basal ganglia and various fibre tracts (MFB/fornix) connecting cortical and ganglia components, the limbic system appears to be predominantly responsible for our emotional life and has a key role in affective and cognitive functioning. An extension of this ‘basic’ limbic system is the mesocorticolimbic system. This extended circuitry chiefly involves dopaminergic projections from the VTA in the midbrain into the limbic system to areas such as the nucleus accumbens, septal nucleus and various cortical regions. It is commonly known as the reward/reinforcement pathway and is traditionally studied in association with addiction (Koob & Nestler, 1997). The nucleus accumbens located in the ventral forebrain, ventral and medial to the caudate, as stated above, is one of the primary terminal projection sites of VTA dopaminergic cell bodies. This key region has been postulated to serve as a gate or filter for information concerned with affect and with certain types of memory projections from the hippocampus to other parts of the brain (frontal cortex and hypothalamus), with dopaminergic projections modulating the flow of neural activity through this filter network (Carlsson et al., 2000; Zmarowski et al., 2005). The mesocorticolimbic system does not exist in isolation; at each level it has critical connections to other brain regions, in particular the basal ganglia (substantia nigra) and other limbic structures (illustrated in Fig 5.11.). Chronic administration of Org 26576 and Org 24448 resulted in activation not only of key components the limbic system, but also of this ‘extended’ circuitry. Areas including the VTA, nucleus accumbens, medial septum and associated areas such as the substantia nigra all exhibited significant activation which was not witnessed with simple acute administration. These effects are important as not only do they show ‘potentiation’ of the functional response from acute to chronic administration, but also the areas activated such as the VTA and nucleus accumbens have been implicated as important brain regions in conditions such as depression and psychoses, previously described briefly in Chapter 4.
Fig 5.11. Diagrammatic representation of the limbic system, with specific emphasis on the components of the mesocorticolimbic components.
5.4.3. Functional effects and current theories

The brain circuitry involved in depression as outlined in Chapter 4 is extensively characterised. Modern antidepressants (second generation SSRI’s) however, are no more therapeutically effective than the compounds that were serendipitously discovered decades ago such as iproniazid and imipramine. In point of fact, electroconvulsive shock therapy is still the most effective treatment for depression. All currently available treatments of depression restore the compromised activity of (corticolimbic) monoaminergic pathways (Delgado, 2000), and over the past decade or so it has become the enshrined view that the proprietary cause of depression is a chemical imbalance specifically in this monoaminergic system (Manji et al., 2003). The monoamine/chemical hypothesis states specifically that mood disorders are caused by a deficiency in serotonin and/or noradrenaline at functionally important receptor sites, causing structural and/or functional changes in the brain. There is mounting evidence however against this rigid, narrow and entrenched viewpoint (Nestler et al., 2002).

Results in this study with chronic administration of both Org 26576 and Org 24448 demonstrate that these novel Ampakines have effects not only in specific, predominantly limbic, brain regions linked with depression but these effects are also rapid in onset, in contrast to current antidepressants, and persist over prolonged periods. Indeed specific effects are seen in the 1) hippocampus, the area thought to mediate the large cognitive component of depression, and an area that brain imaging studies have shown to display abnormalities in blood flow and related measures (Mayberg, 2003), 2) striatum (nucleus accumbens), the area implicated in mediating anxiety decreased motivation and anhedonia, 3) components of the hypothalamus (mamillary bodies), implicated in the neurovegetative symptoms of depression (appetite, energy, sleep and sex), and 4) raphe nucleus/habenula, areas involved in the monoaminergic signalling. All these brain areas operate not only individually but in a series of highly interacting parallel circuits, perhaps suggestive of the existence of a depression ‘neural circuitry’.
The evidence presented above, lends support to a new theory of depression that is garnering increased support. First postulated by Nestler et al. in 2002, the network hypothesis (or systems dysregulation theory) is based on the principle role of the nervous system to store and process information and not the chemical balance of signalling molecules (see Castrén, 2005). Therefore it asserts that mood disorders reflect problems not in individual isolated monoaminergic pathways but in information processing within neural networks in the brain. Therefore, problems in activity dependant neuronal communication may underlie depression and antidepressants, and treatments that alleviate depression may function by improving information processing in these affected neural circuits. It is complementary to the chemical hypothesis. As highlighted above, these novel Ampakines, affect discrete mesocorticolimbic neuronal circuitry intimately associated with affective mood disorders and are thus likely to be eliciting their effects through modulation of this ‘neural’ circuitry via adaptive chemical changes, i.e. glutamatergic signalling resulting in an up-modulation of activity, and/or molecular changes restoring the compromised functionality and re-establishing a normal mood state.

Depressive states are often comorbid with other psychiatric disorders such as schizophrenia, and chronic depression can eventually evolve into psychosis. Schizophrenia, the typical psychosis, is a chronic disorder that develops over time, and is characterised by failures in nearly all aspects of higher-order behaviour: disruption of information processing and sensory perception, abnormal mood and affect, profound cognitive impairment and movement abnormalities. Current understanding dictates that with such a multifactorial heterogeneous disorder/failure of normal behaviour the condition must at least involve areas of the brain such as the frontal cortical systems, limbic system, basal ganglia and thalamus: incidentally all of which are components displaying activation by chronic Org 26576 and Org 24448 treatment. Schizophrenia is also considered by many as the best established of the potential therapeutic targets for glutamatergic intervention. As such, Org 24448 is currently in clinical trails for the treatment of psychosis.
The dopaminergic hypothesis is still considered by many as the leading causative
type on the pathophysiology of schizophrenia (Carlsson, 1995): specifically that
schizophrenia primarily stems from excessive mid-brain dopaminergic
neurotransmission. As a result of this, all current treatments are anti-dopaminergic,
which essentially prevent relapses in schizophrenia whilst managing symptoms with
varying degrees of success. This theory is supported mainly by indirect
pharmacological techniques and brain imaging (PET) (Hietala et al., 1994; Dao-
Costellana et al., 1997). In view of the close interaction between neurotransmitters
in the brain it is highly unlikely that dopamine is the only neurotransmitter to exhibit
dysfunction (Carlsson et al., 2000). The elevated dopaminergic activity in
schizophrenia could possibly be a compensatory response to a functional failure in an
alternate neurotransmitter system. There is an increasing awareness that to move the
status of schizophrenia treatment forward, truly novel therapeutic options, such as
Ampakines, must be considered and that the rationale behind this rigid ‘dopamine’
hypothesis must be questioned.

The major competing causative theory is based around the role of reduced
neocortical glutamatergic functionality in schizophrenia (Goff & Coyle, 2001; Javitt,
2004; Tuominen et al., 2005). Evidence for this is abundant: decreases in,
concentrations of glutamate in the cerebrospinal fluid (Kim et al., 1980);
concentrations of glutamate in the cortex and hippocampus (Tsai et al., 1995); levels
of AMPA and kainate binding in the hippocampus (Kerwin et al., 1988 & 1990);
mRNA levels encoding AMPA subunits and protein levels in the hippocampus, para-
hippocampus, and thalamus are all well documented in schizophrenic patients
(Eastwood et al., 1995 & 1997; Meador-Woodruff & Healy, 2000; Meador-
Woodruff et al., 2001). The most prominent changes are in nuclei with reciprocal
connection to limbic regions, as would be expected. It is also well known
neuroanatomically, that glutamatergic neurones influence dopaminergic
neurotransmission. Indeed reciprocal connections between corticocortical,
corticolimbic and corticothalamic projections are exclusively glutamatergic, and
dysfunction of glutamatergic neuronal systems is not inconsistent with the dopamine
hypothesis of schizophrenia as reciprocal synaptic connections exist between
forebrain dopaminergic connections and glutamatergic systems (Carlsson and Carlsson, 1990). In point of fact glutamatergic neurones can control dopaminergic neurones both directly (excitatory) or via GABA-interneurones (inhibitory). Therefore dysregulation of one system would be expected to alter function in the other, and by enhancing function in one system, by Ampakine treatment, it may be possible to restore some level of function in the other. It should also be noted that typical antipsychotics and clozapine, which are currently in use, are also indicated to enhance glutamatergic neurotransmission.

From a therapeutic standpoint, research has focused on the role of disrupted NMDA receptor function as an ideal mechanism to underlie/underpin the profound behavioural abnormalities witnessed in schizophrenia. Indeed administration of psychomimetic agents (PCP / ketamine) to normal patients results in the production of profound schizophrenia like effects (Pearlson, 1981; Krystal et al., 1994; Olney et al., 1999), and aberrant behaviour in animals (Freed et al., 1980). The AMPA receptor however, as it is an excitatory ion channel that feeds directly into NMDA receptors, could possibly indirectly enhance NMDA function and thus be therapeutically effective in schizophrenia. CX516 for example, synergistically blocked methamphetamine induced rearing behaviour in rats when added to clozapine and to conventional antipsychotic agents (this effect is believed to be predictive of antipsychotic efficacy) (Hess et al., 2003). In human trials, CX516 was added to clozapine again in a placebo-controlled 4–week trial (Goff et al., 2001). The drug combination was well tolerated without any notable side effects, and results illustrated significant improvements in tests of attention, memory and distractibility. The novel Ampakines examined in this study, activate key regions associated with schizophrenia and quite readily support this alternative glutamatergic hypothesis while still incorporating elements of the dopaminergic hypothesis by synergistically/polysynaptically activating dopaminergic areas such as the VTA and substantia nigra. This leads further support to the concept of a ‘brain network’ treatment for CNS disorders rather than the precise targeting of a specific neurotransmitter pathway.
5.4.4. Structural effects and protein levels implicated in Ampakine function

Prolonged depression in humans can result in an ~19% decrease in hippocampal volume (Bremner et al., 2000), and there is a persistence in this hippocampal atrophy post-depression, possibly indicative of neuronal loss (Sapolsky, 2000). Neurogenesis is a natural process in which neural stem cells proliferate and produce new neurones in the adult dentate gyrus of the hippocampus. ‘Classical’ antidepressants such as SSRIs and tri-cyclic’s have been shown to be able to increase neurogenesis in the hippocampus (Duman et al., 1997). This drug-induced cell proliferation requires repeated drug administration and is solely confined to the hippocampus. Increased serotonergic neurotransmission has also been shown to be able to promote hippocampal neurogenesis (Jacobs et al., 2000). Santarelli’s group at Columbia recently intimated that neurogenesis might play a crucial role in the mechanism of anti-depressant drug action. Specifically they suggest that neuronal proliferation may be required for the clinical mood elevating effect of certain antidepressants (Santarelli et al., 2003). Indeed, chronic administration of the biarylsulphonamide AMPA potentiator LY451646 was shown to be able to increase progenitor cell proliferation (by ~45%) in the dentate gyrus of the hippocampus in a dose-dependent manner (Bai et al., 2003). However, in the study outlined in this thesis, both Org 26576 and Org 24448 did not modify neurogenesis in any way following prolonged chronic administration for one and four weeks, even though they exhibited profound functional effects in key brain regions implicated in depression (raphe nucleus, hippocampus, cortex). In addition, the levels of dividing cells in the subgranular zone of the hippocampus were broadly in line with previous reports of ~4500 cells per bilateral mouse dentate gyrus (Nakagawa et al., 2002) for control treated animals. No differences were witnessed in the spatial distribution or morphology of dividing cells.

Much of the current research into AMPA receptor potentiating compounds has focused on the role of neurotrophins such as BDNF, as downstream mediators of their effects. BDNF is abundantly expressed across the central nervous system (Conner et al., 1997) with particularly high expression in the dentate gyrus of the
hippocampus, and has been implicated in brain development, neurogenesis, synaptic transmission and learning and memory (functional roles in which Ampakines have also been implicated). As with neurogenesis, traditional antidepressant treatment increases the levels of brain BDNF, mRNA and protein, in the rodent hippocampus and cortex (Duman et al., 1997), and there is evidence to suggest antidepressants increase BDNF in humans as well (Shimizu et al., 2003; Aydemir et al., 2005); BDNF has also been shown to promote cell proliferation (neurogenesis), survival and/or differentiation both in vivo and in vitro (Pencea et al., 2001). Relevant to the studies described within this thesis, BDNF also has been show to regulate the expression of AMPA receptor subunits and AMPA receptor associated PDZ proteins which in turn regulate AMPA receptor trafficking and stability in neurones (Jourdi et al., 2003); and likewise, there is strong evidence for powerful effects of BDNF in the mesocorticolimbic system (VTA-NAc pathway) (Horger et al., 1999). The so-called ‘neurotrophin’ hypothesis states that repetitive neuronal activity enhances expression, secretion and or action of neurotrophins at the synapse to modify synaptic transmission and connectivity possibly in this case via reciprocal actions between BNDF and AMPA receptors. This provides convenient connection between neuronal activity and synaptic plasticity (Schinder and Poo, 2000). In fact, exogenous application of BDNF results in antidepressant effects, and increases in BDNF are observable in chronic antidepressant treated mice/humans (Siuciak et al., 1997; Mackowiack et al., 2002). BDNF has also demonstrated the ability to promote the function, sprouting and re-growth of 5-HT containing neurones in the brains of adult rats. Compounds therefore which can potentially up-regulate BDNF could in essence promote monoamine-containing neurone growth and function in a ‘deficient’ system (Altar, 1999).

Specifically with Ampakines however, the evidence pertaining to functional BDNF involvement is limited. CX546 (250 μM) increases BDNF expression (mRNA), in a refractory manner, in vitro by 5.5 fold, but in vivo (40 mg/kg) only produces slight increases in BDNF cRNA (Lauterborn et al., 2000 & 2003). Also, excessively high concentrations of the currently available CX compounds are required to induce BDNF expression levels, and the supporting evidence indicate that the magnitude of
BDNF protein changes are marginal. Org 24448 has virtually no effects on BDNF induction (translation or protein) in *in vitro* studies carried out so far. The biarylpropylsulfonamides (LY451645) on the other hand increase both BDNF mRNA (25 fold) and protein (7 fold) levels *in vitro* and *in vivo* (Legutko and Scholnick, 2001; Mackowiack *et al*., 2002), and concomitantly actively increase progenitor cell proliferation in the rat hippocampus (Bai *et al*., 2003). It is entirely feasible that the AMPA-BDNF-neurogenetic pathway plays a role in the functionality of biarylpropylsulfonamides, but is unaffected by benzodithiazide compounds such as Org 26576 and Org 24448, as demonstrated in this study.

These ‘structural’ phenomena highlighted above are all tightly regulated by interactions with a series of intracellular signals and transcription factors. The mitogen-activated-protein-kinases, ERK1/2 for example, play a major role in the modulation of neuronal growth and differentiation (neurogenesis) and have also been implicated in AMPA receptor functioning and signalling *in vitro*, as is Lyn kinase. BDNF expression is also regulated by Lyn-mediated activation of MAPK in response to AMPA receptor signalling (Hayashi *et al*., 1999). CX516 for example has been shown to activate ERK1/2 pathways in the CA1 region of the hippocampus specifically via the AMPA receptor (as NMDA blockage has no effects) (Bahr *et al*., 2002). Another signalling correlate common in AMPA/ERK function is the cAMP-response-element-binding protein (CREB), which has also been implicated as playing a major role in the aetiology of depression and psychoses. CREB mRNA is induced by antidepressants (Thome *et al*., 2000; Duman *et al*., 2000 & 2002), and overall levels of CREB and phosphorylated-CREB are reduced in post-mortem brain tissue of depressed and psychotic patients (Dowlatshahi *et al*., 1998). Moreover, CREB activates the production of BDNF, which is discussed above. In addition to examining the effects of chronic administration of Org Ampakines on these ‘downstream’ effectors of AMPA, the levels of AMPA receptors was also investigated, as there is evidence from *in vitro* studies that prolonged Ampakine application results in degradation/internalisation of AMPA receptors due to over-stimulation (Jourdi *et al*., 2005a/b). However, in this study no changes were witnessed both in receptor protein levels (GluR1-2-3) and in downstream signalling.
molecules such as P-CREB, Lyn-Kinase, Calbindin and the MAPKinas, ERK1-2, with 7 or 28 days Ampakine treatment.

5.4.5. Technical considerations
The reason no significant effects on structural correlates were seen with chronic novel Org administration is not easily explained. The functional effects witnessed may be purely due to kinetic effects of the compounds on the target AMPA receptors. AMPA receptors are known to undergo rapid desensitisation and rapid recycling in the membrane, and glutamate itself has a relatively low affinity for AMPA receptors. Ampakines themselves also have a very fast on/off rate in relation to the receptor, and therefore the functional LCGU changes witnessed may be due to constant low level ‘priming’ of the AMPA receptor increasing functional activity and may not be accompanied by changes in gross down-stream signalling / protein levels. However appealing this concept is it is highly unlikely. A more parsimonious explanation as to why no effects on neurogenesis or protein levels are seen is the validity of the dosing regime. We know however that both Org 26576 and Org 24448 exhibit good blood-brain-brain penetration and that the doses chosen have profound functional effects (LCGU) in the CNS. This study also specifically investigated the ability of the novel Ampakines to promote total cell differentiation after a prolonged period of drug administration. An alternative to this would be to examine the ability of the drugs to promote the survival of newly born cells by administering an initial BrdU injection followed by chronic administration of the compounds. The drugs therefore may stabilise the long-term survival of newly born cells, not promote their proliferating numbers. This would require further investigation.

As far as the neutrality of the results with regard to protein levels, again there are several feasible explanations. The time points chosen in this study for example may not be appropriate to pick up changes in signalling molecules. Changes in ERK and P-CREB may in reality occur within a few days of administration (acute phase) and may therefore be overlooked. In addition, the sampling of tissue, i.e. whole hippocampi, may not be sufficient to identify discrete, possibly regional changes, especially in GluR levels. The overall levels of receptors may not be changing as
witnessed, but their dynamics may be changing with respect to cycling within the cell membrane, which unfortunately cannot be identified with the current assay. Examining the receptors, for example, in both the membranous and cytosolic protein fractions could allow the discrimination of any discrete changes. It may also be the case that proteins, other than those investigated in this thesis, could play a role in Ampakine function. It would be highly instructional for example to investigate AMPA interaction protein such as GRIP/ABP/PICK, intracellular proteins which play key roles in AMPA receptor cycling and cellular targeting, to help to further understand AMPA receptor subunit movements in response to Ampakine treatment (Dong et al., 1997; Srivastava et al., 1998; Dev et al., 1999). Modulatory protein such as stargazin (Letts et al., 1998), a natural AMPA receptor potentiator (Yamazaki et al., 2004; Priel et al., 2005), and interactions with G-proteins could be investigated; as AMPA receptor signalling in some neurones has been reported to involve a G-protein coupled to protein kinase cascades (e.g. Ras, Raf) (Wang & Durkin, 1995).

5.4.6. Summary

The present study is the first to demonstrate that chronic administration of both Org 26576 and Org 24448 results in rapid and prolonged increases in functional activity in key regions of the mouse cerebrum. Chronic administration resulted in activation of brain regions previously witnessed in the acute study with recruitment of extra regions including several brain stem and forebrain nuclei such as the ventral tegmental area of Tsai (VTA), nucleus accumbens, prefrontal cortex, and septum. These regions are implicated with key CNS dysfunctions such as depression and psychoses and are intimately interlinked in complex neural networks, lending support to the concept of neural-networks as relevant targets for disease intervention. Chronic administration of the novel Org compounds in no way effected hippocampal neurogenesis, or associated receptor/signalling proteins, suggesting that these novel Ampakines elicit their effects in a neurogenetic independent manner.
Chapter 6
Modulation of AMPA receptor kinetics by Org 26576 and Org 24448 influences synaptic plasticity in the murine hippocampus
6.1. Introduction

The hippocampus is a key part of the limbic system, and therefore plays an important role in the emotional aspects of behaviour. Additionally, it is recognised as a crucial neural component in the formation and storage of memory. It is however extremely susceptible to various neurodegenerative pathologies, and as such, ‘cognitive’ deficiencies are often associated with ‘affective’ conditions such as depression, various psychosis, Alzheimer’s and Parkinson’s disease. This thesis has previously illustrated that both acute and chronic administration of the novel Ampakines Org 26576 and Org 24448, induce profound functional increases in activity, which are especially evident/prominent in key components of the hippocampal architecture.

AMPA receptors, which are abundantly expressed in the hippocampus, play a crucial role in the induction and stabilisation of long term-potentiation (LTP), currently the leading experimental mechanism to explain the process of memory formation (see section 1.6.2.2.). This phenomenon has been studied extensively using the hippocampus as a model due to its precisely organised structure and the ease with which defined pathways can be stimulated. It is proposed that positive modulation of AMPA receptors, facilitates LTP induction and consequently enhancement of memory function/formation. Indeed, CX516 and CX546, two first generation Ampakines, have been shown to potentiate LTP both in hippocampal slices in vitro and in freely moving conscious animals in vivo (Staubli et al., 1994a/b). In conjunction with this, Ampakines also facilitate memory formation in animal behavioural models, such as the Morris water maze and can reverse pre-existing memory impairments (Sekiguchi et al., 2001). This in turn, leads to the proposition that AMPA receptor potentiators could be of potentially therapeutic relevance, as ‘cognitive enhancers’, treating the memory impairments in conditions associated with a cognitive deficit.

6.1.1. Aims

The aim of the current study was to establish if the novel Ampakines Org 26576 and Org 24448 affected AMPA receptor function in acute mouse hippocampal slices; and more importantly, whether this resulted in changes in the magnitude of LTP.
6.2. Materials and Methods

6.2.1. Animals and slice preparation
C57Bl/6J male mice (8-10 weeks old) were communally housed on a 12 hr light/dark cycle for 1 week prior to experimentation with free access to food and water. On the day of experimentation a single mouse was isolated, weighed and halothane anaesthetised. Acute hippocampal brain slices were prepared as previously described (Section 2.8.2.).

6.2.2. Electrophysiological recordings
After 60 minutes in the holding chamber, slices were transferred into the submerged slice recording chamber and extracellular recordings were made from the CA1 subfield of stratum radiatum as previously described (Section 2.8.4.). Evoked responses (see below) were amplified, filtered, digitised, and the resultant data was stored and analysed as previously described on a personal computer using Signal data acquisition and analysis program (Section 2.8.5.).

6.2.3. Stimulation protocols
Baseline synaptic responses were generated by stimulation of the Schaffer collateral/commissural afferents every 20 s. In all experiments the input-output relation of the synaptic response was first established to determine the maximum fEPSP amplitude attainable without a population spike component. The stimulation was adjusted to obtain 35% of this maximum fEPSP amplitude and remained fixed thereafter. For basic dose-response effects on amplitude, a stable baseline was established for at least 10 min, and the perfusion line was then switched to aCSF containing Org 26576 or Org 24448 at the test concentration. For LTP experiments slices were disinhibited with the addition of 10 μM Bicuculine, a specific GABA₄ antagonist, and were also subjected to a CA3/CA1 cut to remove CA3 input and prevent orthodromic epileptiform activity. The stimulus intensity was again set to obtain 35% of the maximum EPSP amplitude, and a stable non-drug baseline was obtained for at least 10 min. Following this, the perfusion system was switched to aCSF containing the appropriate concentration of Org compound and a stable baseline in the presence of drug was obtained for a further 10 min (the stimulus
intensity was not decreased to bring the response amplitude back down to pre-drug levels), as this would have reduced the population of activated nerve fibres. LTP was induced using standard high frequency stimulation (HFS), consisting of four stimulation trains separated by 20 s intervals. Each train consisted of 25 pulses at a frequency of 100 Hz and was delivered at the same intensity as that used to evoke baseline responses. Following HFS, fEPSPs were recorded every 20 s.
6.3. Results

6.3.1. Effects of the Ampakine CX516 on fEPSP kinetics
The effects of CX516 on fEPSP amplitude are shown in Fig 6.1.B-D. CX516 (1 mM) had pronounced effects on both amplitude and half-width, increasing each by 45.17 ± 3.2% (SEM, n = 4) and 39 ± 2.6% respectively. The effects of CX516 were relatively rapid in onset (maximal effect within 15 min) and were quickly reversed during washout with normal aCSF to pre-drug baseline levels (Fig 6.1.A).

6.3.2. Effects of the novel Ampakines Org 26576 and Org 24448 on baseline fEPSP responses in acute hippocampal slices
The two novel Ampakines Org 26576 and Org 24448 increased fEPSP amplitude (Figures 6.2. & 6.3.). Org 26576 (Fig 6.2.) increased fEPSP amplitude in a classical dose-dependent manner, over a two log unit concentration range, from 3 μM (4.1 ± 0.35% increase in amplitude) to 300 μM (38.99 ± 0.17% increase). Org 26576 had no effect on the half-width of the response. Similarly, Org 24448 caused an increase in fEPSP amplitude over a wide concentration range (Fig 6.3.). At 3 μM, fEPSP amplitude was increased by 8.46 ± 0.17%, while the largest increase in amplitude, 143 ± 5.28%, was elicited by 1mM Org 24448. Org 24448 at all concentrations tested had no effect on the fEPSP half-width.

Comparing the two compounds, Org 26576 elicited an increase in fEPSP amplitude that did not exceed 38% over a lower more ‘compact’ dose range with an EC50 for amplitude of 20 μM; whilst Org 24448 produced a large increase in amplitude (140% maximum increase) over a much wider dose range, with an EC50 of 225 μM. The onset of drug action was rapid for both compounds with a maximum response obtained within ~10 min; reversal of drug action to pre-drug response levels was also fast, in a manner similar to CX516. Addition of CNQX (20 μM), a specific AMPA receptor antagonist, to the aCSF, in the presence of either Org 26576 or Org 24448, resulted in the total block of the fEPSP (Fig 6.4.A.).
Fig 6.1. Effects of the prototypic Ampakine CX516 on fEPSP kinetics

1mM CX516 application resulted in an average increase in amplitude of 45.17% ± 3.2, and an average increase in half-width of 39% ± 2.6 (n=4). CX516 (1 mM) has rapid effects on amplitude and equally is rapidly washed off to baseline levels. Representative traces of the baseline fEPSP (B) and subsequent 'enhancement' with addition of 1 mM CX-516 (C) are also shown. The responses are shown in overlay (D) (The dotted line is representative of the 'stretched' baseline response to the corresponding amplitude increase witnessed with 1 mM CX516, the effects on half-width is clearly visible). Scale bar = 0.2 mV by 5 msec.
Fig 6.2. Dose-response effects of Org 26576 on fEPSP amplitude

Increasing concentrations of Org 26576 induce dose-dependant increases in fEPSP amplitude, with a maximal increase in amplitude of ~38% in response to 300 \( \mu \text{M} \) Org 26576 (A). EC\(_{50}\) for amplitude response = 20 \( \mu \text{M} \). No effects on fEPSP half-width were witnessed at any concentration. Data are presented as average \( \pm \) SEM (n=3 per concentration). Representative traces of the effect of Org 26576 at 10 \( \mu \text{M} \) (B) 30 \( \mu \text{M} \) (C) and 300 \( \mu \text{M} \) (D) are shown superimposed over their corresponding baseline responses (The dotted line in (D) is representative of the 'stretched' baseline response to the corresponding amplitude increase witnessed with 300 \( \mu \text{M} \) Org 26576). Scale bar = 0.25 mV by 5 msec.
Fig 6.3. Dose-response effects of Org 24448 on fEPSP amplitude

Increasing concentrations of Org 24448 induce dose-dependent increases in fEPSP amplitude, with a maximal increase in amplitude of ~140% in response to 1 mM Org 24448 (A). EC₅₀ for amplitude response = 225 μM. No effects on fEPSP half-width were witnessed at any concentration. Data are presented as average ± SEM (n=3 per concentration). Representative traces of the effect of Org 24448 at 3 μM (B) 250 μM (C) and 1 mM (D) are shown superimposed over their corresponding baseline responses (The dotted line in (D) is representative of the ‘stretched’ baseline response to the corresponding amplitude increase witnessed with 1 mM Org 24448). Scale bar = 0.25 mV by 5 msec.
Addition of 20 μM CNQX to the perfusing aCSF completely blocks the fEPSP response in both Org 26576 and Org 24448 drug-treated (A) and LTP induced (B) acute hippocampal slices. Data are presented as average ± SEM (n=6) for Org treated slices, and a representative trace is shown for the effects on LTP. Representative traces of fEPSP responses are shown individually before (i) and after (ii) CNQX administration; and in overlay (iii). Scale bar = 0.2 mV by 5 msec.
6.3.3. Stable LTP induced by high-frequency-stimulation (HFS)

Long-term-potentiation (LTP) was induced in the disinhibited hippocampal slice using a HFS paradigm (Fig 6.5.). Having established a stable baseline response for at least 10 min, HFS induced an immediate potentiation of the fEPSP. This ‘post-tetanic peak’ was evident as a 125% increase in fEPSP amplitude. This initial potentiation of fEPSP amplitude decayed rapidly from this peak over the following 10 min, and eventually stabilised for the remainder of the experiment at approximately 55% above the initial baseline. This degree of LTP was not only consistent, as attested by the small standard error of the potentiation, but was also not the maximum potentiation attainable. If at 60 min post-induction, a further two trains of 25 pulses at 100Hz pulses were given, the level of LTP could be raised even further (~80%) above baseline. This result is important, as it highlights the fact that the response studied here had not reached a ceiling and could be up and down regulated by drugs. Addition of CNQX (20 μM) when a stable level of LTP had been achieved resulted in the abolition of the entire fEPSP (Fig 6.4.B.).

6.3.4. Effects of ‘physiologically relevant’ concentrations of Org 26576 and Org 24448 on LTP

Concentrations of Org 26576 and Org 24448 that corresponded to in vivo doses used previously in the 2-deoxyglucose studies, and had previously been measured in blood samples / microdialysis studies from intraperitoneally injected animals (Organon - personal communication) were chosen to be tested the LTP model.

Both Org 26576 (3 μM) and Org 24448 (10 μM) significantly potentiated the initial post-tetanic peak following HFS, to ~175% over baseline (Figures 6.6. & 6.10.). Org 26576 (3 μM) increased the stable level of LTP from 55% to 90% over baseline for the duration of the experimental period, effectively facilitating the maximal level of LTP attained. Org 24448 (10 μM) on the other hand, after the initial enhancement of the post-tetanic peak, displayed a decrement in the response amplitude which eventually stabilised at control levels ~20 min post-potentiation. It is also interesting to note that LTP induced in the presence of Org 24448 (10 μM) resulted in an obvious modification of the fEPSP waveform in the post-tetanic phase (Fig 6.7.).
Fig 6.4. Stable LTP induced by HFS in acute hippocampal slices

A ‘classical’ stable model of LTP induced by HFS was achieved by bath saturation with 10 μM bicuculline, a GABA_A receptor antagonist, and by cutting to remove input from the CA3 to prevent isotonic epileptiform activity (A). Data are presented as average ± SEM (n=8). Representative traces of fEPSP responses are shown individually prior to (B), immediately after (C), and 60min post-LTP induction (D). Responses prior to LTP and 60min post-LTP are also shown in overlay (E). The response is clearly still potentiated 60min post-induction. Scale bar = 0.2 mV by 5 msec.
This was manifested as epileptiform-like activity accompanied by a positive-going late component of the fEPSP that would correspond to a hyperpolarisation in an intracellular recording. This was unexpected, as great care had been taken to sever the CA3 input to prevent isotonic epileptiform activity that might be expected following disinhibition of the slices; the implication of this observation is that this aberrant activity originated in area CA1 itself as a result of exposure to Org 24448, because no ‘spiking activity’ was seen in control or Org 26576-treated slices (Fig 6.7.).

### 6.3.5. Effects of EC$_{50}$ concentrations of Org 26576 and Org 24448 on LTP

The EC$_{50}$ concentrations of Org 26576 and Org 24448, for effects on fEPSP amplitude were 20μM and 225μM, and this concentration for each compound was investigated for action on LTP.

Neither compound had any significant effect on the initial post-tetanic peak although both had dramatic and unexpected actions on LTP (Fig 6.8.). Unlike ‘physiological’ concentrations, both compounds to differing degrees ‘inhibited’ LTP formation. Org 26576 (20 μM) reduced the stable level of LTP from 55% to 25% over baseline for the duration of the experimental period, the response stabilising at this lower level ~30 min post-potentiation. Org 24448 (225 μM) on the other hand, after the initial post-tetanic peak, caused a rapid decrement in response, which continued to decay for the remainder of the experimental period until it eventually reached the pre-HFS baseline amplitude at 60 min post-potentiation. LTP induced in the presence of Org 24448 (225 μM) resulted in the same obvious modification of the fEPSP waveform immediately post-potentiation in a manner similar to that seen with ‘physiological’ concentrations of Org 24448 (Fig 6.9.). No such ‘spiking activity’ was seen in control solutions or in Org 26576 (20 μM) treated slices.
Fig 6.6. Effects of physiologically active concentrations of Org 26576 and Org 24448 on LTP

3 µM Org 26576 results in both a pronounced increase in the immediate post-tetanic potentiation (B) and an ~50% increase in the stable ‘ceiling’ of induced LTP 60 min post potentiation (D) when compared to control responses (red circles). This potentiation is significantly greater than control from 7 min post-potentiation. 10 µM Org 24448 (yellow triangles) application results in a similar increase in the post-tetanic peak (B); but unlike Org 26576 the response amplitude gradually decayed to control LTP levels by 11 min post-potentiation. Data are presented as average ± SEM (n=3/4 for each compound, with interleaved controls).
Fig 6.7. Representative traces from LTP experiments with the addition of 'physiologically active' concentrations of both Org 24448 and Org 26576.

Representative fEPSPs recorded during the baseline drug response (A) (average of 10 traces), immediately (B), 30min (C) and 60min (D) post-induction of LTP (raw). Traces (A) and (D) are also shown in overlay (E). Note the pronounced 'epileptiform' activity induced post-HFS with Org 24448 (10 μM) even with a prior CA1/CA3 cut, followed by hyperpolarisation. Scale for both Org 24448 and Org 26576 traces = 0.5 mV by 5 msec.
Fig 6.8. Effects of EC$_{50}$ (amplitude) concentrations of Org 26576 and Org 24448 on LTP

Both compounds elicit an immediate post-tetanic potentiation (B) of a comparable size to that of the control (red circles). However, whereas the control LTP stabilises ~10 min post induction and is stable for the following 50 min, both Org 26576 (blue squares) and Org 24448 (yellow triangles) exhibit a pronounced decrement in response amplitude over the experimental time-course, which is significant for Org 24448 12 min post-potentiation, and Org 26576 30 min post-potentiation. The response amplitude for Org 24448 decays to baseline by 60 min post-potentiation, whilst Org 26576 is stably potentiated after 60 min but at a lower level compared with controls. Data are presented as average ± SEM (n=3/4 for each compound, with interleaved controls).
Fig 6.9. Representative traces from LTP experiments with the addition of EC$_{50}$ concentrations of both Org 24448 and Org 26576

Representative fEPSPs recorded during the baseline drug response (A) (average of 10 traces), immediately (B), 30 min (C) and 60 min (D) post-induction of LTP (raw). Traces (A) and (D) are also shown in overlay (E). The ‘epileptiform’ activity induced post-HFS with Org 24448 (225 μM) is even more pronounce at this higher concentration, and is again followed by a pronounced hyperpolarisation. Scale for Org 26576 = 0.25 mV by 5 msec; Org 24448 (A,C,D,E) = 0.20 mV by 5 msec; (B) = 0.5 mV by 5 msec.
Fig 6.10. Effect of physiological (A) and EC$_{50}$ (B) concentrations of Org 26576 and Org 24448 on the initial post-tetanic peak

Physiological concentrations of both Org 24448 (10 μM) and Org 26576 (3 μM) significantly increased the size of the immediate post-tetanic peak (Paired students t-test, Org 24448 P=0.007; Org 26576 P=0.035). EC$_{50}$ concentrations of both compounds (225 μM and 25 μM respectively) had no significant effect on the post-tetanic peak. Data are presented as average ± SD.
6.4. Discussion

The primary aim of this study was to characterise the effects of the novel Ampakines Org 26576 and Org 24448 on AMPA receptor-mediated synaptic responses using extracellular field recordings from acute hippocampal slices. A further aim was to determine whether these compounds were able to modify LTP, a much-studied form of synaptic plasticity. The results presented here clearly demonstrate that both compounds increase the strength of excitatory synaptic transmission, as measured by fEPSP amplitude, directly via the AMPA receptor, without effecting fEPSP half-width. Physiologically relevant concentrations of the compounds potentiated a stable form of classical LTP, whilst EC$_{50}$ concentrations had the effect of preventing the maintenance of LTP, resulting in less marked potentiation that was characterised by a time-dependent decline (particularly in the case of Org 24448).

6.4.1. CX516, Org 26576 and Org 24448 modify extracellular field excitatory post-synaptic potentials

The use of Ampakines in electrophysiological studies to assess their effects on unpotentiated synaptic responses, deactivation/desensitisation kinetics, and models of synaptic plasticity such as LTP and LTD, are well documented in the literature. As early as 1990 Ito and colleagues showed that aniracetam, an early AMPA receptor potentiator, acted by selectively enhancing AMPA-receptor-mediated responses, in both *Xenopus* oocytes injected with AMPA receptor mRNA and hippocampal CA1 pyramidal cells. The prototypic Ampakine, CX516, has been examined extensively in electrophysiological studies and its effects on extracellular field recordings are well documented. CX516 (1 mM) was utilised in this study as a positive control and was found to produce rapid effects on fEPSPs, which were fully reversible, with maximal increases in amplitude of 45% and half-width of 39%. Previous reported effects for 1 mM CX516 on fEPSP amplitudes in acute *rat* hippocampal slices include increases of 43% and ~50%, and for half-width reported values range from ~30% to 44% (Arai *et al*., 1996 & 2002 & 2004). The results obtained in this study are comparable with these values, highlighting the validity of the acute *mouse* hippocampal slice model utilised in these experiments, and allowing subsequent comparison between published work and results obtained with Org 26576 and Org 24448.
The present study also demonstrates that Org 26576 and Org 24448 exhibit a clear dose-dependent effect on the amplitude but not on half-width of evoked fEPSP responses. Org 26576 produced a maximal increases in amplitude of 38% (300 μM) with an EC₅₀ concentration of 20 μM with a significant increase seen at concentrations as low as 3 μM. Org 24448 produced a maximal increase in amplitude of 140% (1 mM) with an EC₅₀ concentration of 225 μM and with significant increases in amplitudes seen with concentrations as low as 3 μM. The increases in fEPSP amplitude induced by these novel Org compounds were directly AMPA mediated as they could be eliminated by blockade of the receptor with the specific AMPA antagonist CNQX. These results are interesting as both Org compounds specifically affect the amplitude of the response without affecting the half-width. This is unusual as most previous reports investigating ampakine actions on fEPSPs show modification of both amplitude and half-width. CX516, for example, on average increases amplitude consistently between 43 to 50% and half-width by 30% to 45% (Arai and Lynch, 1998; Black et al., 2000; Lin et al., 2002; Arai et al., 2004), whilst CX546 has an opposite effect, increasing amplitude by only 28% but half-width by an impressive 115% (Arai et al., 2002). This difference in effect on the unpotentiated fEPSP may reflect more complex effects on synaptic plasticity. CX516 for example, can promote short-term-potentiation (STP) into LTP, whilst CX546 can both promote STP into LTP and raise the maximum level of LTP induced. This difference could be in part due to their differential effects on baseline kinetics.

6.4.2. Cognition and synaptic plasticity

Cognition can be defined as the mental process of knowing, including aspects such as awareness, perception and judgement. The neuroanatomical basis of memory/cognition is thought to include vital brain areas such as the hippocampus and associated cortical regions, which are important in the encoding, storage, consolidation and retrieval of memories. Coincidentally these ‘key’ areas also exhibit pronounced activation by Org 26576 and Org 24448 following both acute and chronic administration in functional studies (Chapters 4&5).
6.4.2.1. LTP

Long-term-potentiation (see Introduction 1.5.2.), a form of synaptic plasticity, has gained ascendancy as the pre-eminent model for investigating the molecular mechanism of memory. Ionotropic glutamate receptors (AMPA and NMDA) play a pivotal role in the induction and maintenance of LTP, and as such, the process is potentially highly modifiable by compounds, such as ampakines, that modulate receptor kinetics. Indeed intra-hippocampal injection of the AMPA receptor antagonist LY326325 reduces functional activity in the hippocampus (<LCGU) and impairs memory in spatial learning and retention behavioural tests (Micheau et al., 2004). Arai (Arai et al., 1992) was the first to show that ampakines could effectively enhance and stabilise LTP under less than optimum conditions, findings that were replicated with various other drugs (IDRA-21, BDP and CX546) both in vivo and in vitro (Arai et al., 1996). In concurrence with this, Ampakines have also been shown to effectively enhance various forms of memory in behavioural paradigms involving both animals and humans. High-frequency-stimulation (HFS)-induced LTP in the disinhibited acute mouse hippocampal slice was established in this study as a model system and a stable potentiation ~60% above baseline was observed. Other stimulation protocols had been investigated, including theta-burst stimulation (Arai et al., 2004), a paradigm based on a naturally occurring stimulation pattern occurring at theta frequency in vivo during learning. However, due to the high quality of the slices, and thus preservation of inhibitory inputs, and the fact that younger mice have a greater innate inhibitory tone, it was difficult to establish a stable model of LTP using theta-burst stimulation. A ‘classical’ response was obtained by isolating the excitatory pathway by inhibiting the feed-forward inhibitory GABAergic interneurones, which are concomitantly stimulated with the excitatory pathways during HFS, and provide an inhibitory ‘clamp’ on potentiation.
6.4.2.2. Org 26576 and Org 24448 bidirectionaly modify LTP

Even though Org 26576 (3 μM) and Org 24448 (10 μM) at ‘physiological’ concentrations had minimal effects on unpotentiated baseline fEPSP amplitude (5% and 17% increases in amplitude respectively), both produced observable effects on LTP. Furthermore, both potentiated the immediate-post tetanic peak. Org 26576 increased the stable level of LTP by 63% whilst Org 24448 only transiently enhanced LTP and was indistinguishable from control 20 min post-potentiation. The converse effect was witnessed with pharmacological EC\textsubscript{50} concentrations of Org compounds. Org 26576 (20 μM) reduced the stable level of LTP by ~30% but did not completely abolish the potentiation, as was the case with Org 24448 (225 μM) over the course of the 1 hr experimental period.

The extent of depolarisation during induction is believed to be the most critical factor for the activation of NMDA receptors and hence the overall magnitude of LTP (Xia \textit{et al.}, 2005). As the AMPA receptor is the key component contributing to the initial depolarisation, it can be assumed therefore with low physiological doses of Org 26576 and Org 24448 there is essentially a ‘priming’ of the AMPA receptor, enhancing current flow. This in turn produces a larger and faster depolarisation, which has concomitant effects on relief from the Mg\textsuperscript{2+} block of the NMDA receptor, resulting in a greater overall potentiation. It seems that Org 24448 may exhibit this transiently, as manifested by the immediate enhancement of the post-tetanic peak, but the overall level of enhanced depolarisation may not be sufficient (reach a sufficient threshold) to result in stable long-lasting enhanced potentiation. Neurones are also capable of modulating synaptic strength by regulating the number of receptors at the synapse, and increased AMPA receptor trafficking and recruitment to activated synapses is also believed to be a key factor in the induction and maintenance of LTP (see Introduction 1.5.3.). The higher (EC\textsubscript{50}) concentrations of Org 26576 and Org 24448 may have the effect of over-stimulating the AMPA receptors, resulting in a blockage of the recruitment of extra-synaptic AMPA receptors to the stimulated synapse, a reduction in the response amplitude, and ultimately as a result of this a lack of stable enhanced potentiation. Another possible explanation is that trafficking of extra AMPA receptors isn’t inhibited, but that receptors at the active post-synaptic
site are being internalised, due to over-activation caused by the high ampakine concentrations, more rapidly than they are being inserted, resulting in an overall decrease in AMPA receptor levels. This concept is supported indirectly by recent in vitro studies from Julie Lauterborn’s lab (Jourdi et al., 2005a/b), in which cultured hippocampal slices subjected to chronic treatment with ampakines (CX614) at relatively low concentrations (50 μM) exhibited decreased receptor responses to synaptic stimulation, an effect which reflected reduced AMPA receptor surface expression due to calpain-dependent proteolysis of SAP97 and GRIP1; two key AMPA receptor interacting proteins responsible for anchoring the receptor in the membrane.

An unexpected consequence of Org 24448 on synaptic transmission in the LTP model was the manifestation of pronounced epileptiform-like activity accompanied by hyperpolarisation in the late phase of the fEPSPs. Without further experimentation the exact cause of this cannot be accurately determined, but a number of reasons may be hypothesised. Epileptiform activity is largely mediated by a synchronous synaptic activation of cells in local cortical circuits (CA1 in this case), and is presumably associated with a large release of glutamate, which occurs following HFS. With the combination of the local GABAergic system blocked by bicuculline (GABA_A antagonist) and a CA3 cut to prevent feed-forward epileptiform activity into the CA1, Org 24448 which has pronounced effects on the fEPSPs even at low doses, could be causing hyperexcitability in the CA1 which manifest as this localised epileptiform activity. This reasoning is supported by the fact that the effect is more pronounced with higher concentrations of Org 24448 (Higher concentrations = greater activation = enhanced excitability).

This epileptiform activity could also possibly be due to drug stimulation of any residual direct perforant pathway input into the CA1, or by non-specific drug effects on various channels/receptors in the CA1 field. T-type calcium channels (Cav3.1/3.3), which are abundantly expressed in the brain especially the hippocampus, are involved in pacemaking and the controlling of repetitive neuronal firing (Catterall et al., 2005). Non-specific effects of the drugs increasing T-channel
bursting activity could produce epileptiform activity in the CA1 (Czapinski et al., 2005). A combination of hyperexcitability induced by Org 24448 and a lack of inhibitory input resulting in a block of after-hyperpolarisations mediated by Ca\(^{2+}\)-activated K\(^{+}\) channels (via direct channel block) could also result in localised epileptiform activity. M-type K\(^{+}\) channels (K\(_{V}7.2/7.4\)) which determine sub-threshold excitability of neurones are also highly prevalent in the hippocampus, and blockage of M-current can also result in epileptiform activity (Pena and Alavez-Perez, 2006).

### 6.4.3. Future experimental approaches

Electrophysiologically there is great scope for further experimentation with these novel ampkaines to investigate their mode of action. Basic work investigating the compounds effects in two-electrode voltage-clamp recordings from *Xenopus* oocytes with homomeric and heteromeric combinations of expressed AMPA receptors would help determine subunit/receptor combination specificity, which would be highly informative especially in terms of selective drug targeting for specific brain regions. Outside-out voltage-clamp patch experiments with rapid (1ms) and prolonged exposure to glutamate in combination with Org 26576 and Org 24448 would also help delineate any differential effects on desensitisation/deactivation kinetics the compounds may exhibit.

With respect to synaptic plasticity, specifically LTP, the effects of the compounds in more physiologically relevant forms, such as theta burst stimulation induced LTP, would also be informative. More importantly, testing the compounds in a model of compromised functionality (representative of a disease state), such as the aged APOE mouse, which have age-dependent deficits in LTP accompanied by observable behavioural dearth, would prove invaluable in establishing potential therapeutic efficacy in models of actual disease states. An alternative approach to recording monosynaptic responses is to examine ampakine effect in polysynaptic circuits (stimulate DG→CA3→CA1 record), as it has been previously shown that AMPA receptor modulators have a greater influence on synaptic transmission after long chains of connections (Servio et al., 1996); and this approach is also functionally
more relevant in terms of whole brain physiology. It would also be worthwhile investigating the effects of the compounds on LTD, the opposing process to LTP, and if possible investigate the effects of the compounds on AMPA receptor trafficking and recycling in real-time, possibly via GFP tagging of AMPA receptors (Doherty et al., 1997).

6.4.4. Summary
This is the first electrophysiological study to investigate these novel Org ampakines and it has been demonstrated that not only do they enhance the unpotentiated fEPSP but also they bi-directionally modify LTP in a manner that is concentration dependent. Both Org 26576 and Org 24448 produced dose-dependent increases in fEPSP amplitude without having any effect on the half-width of responses, and physiological concentrations of both drugs potentiated a stable form of LTP, with Org 26576 producing a greater more stable potentiation. These results are suggestive that these novel ampakines are effective in boosting the neural correlate of cognition, LTP, and as such will be predictably effective in treating cognitive aspects of conditions such as depression, schizophrenia, Alzheimer’s and Parkinson’s diseases.
Chapter 7

General Discussion
Evidence in the literature has implicated AMPA receptor potentiating compounds as prospective therapeutic agents in the treatment of neurological disorders such as depression, psychoses and cognitive impairment. The data presented in this thesis, investigating the functional effects of the novel Ampakines Org 26576 and Org 24448, provides the first substantive indication of their anatomical basis of action in the CNS following both acute and chronic administration, and highlights their ability to positively modulate AMPA receptor kinetics in the hippocampus. The experiments successfully address all the aims laid out in the Introduction (see section 1.10.), and are supportive of a potential role of these compounds in the treatment of the various conditions outlined above.

7.1. Summary of findings

A semi-quantitative $[^{14}C]$-2-deoxyglucose autoradiographic model for investigating cerebral function was successfully established in C57Bl/6J mice. The model was subsequently shown to be both consistent with previously published data (Kelly et al., 2002) and reliably reproducible. The NMDA receptor antagonist, MK-801 (0.5 mg/kg), when investigated in the model produced ‘classical’ effects on cerebral function, providing further validation. The prototypic Ampakine CX516, when trialled at two doses (30 & 100 mg/kg) had minimal effects of cerebral function, with the higher dose seemingly inducing a catatonic state in the mice. Finally, a pilot study with the Ampakines Org 26576 and Org 24448 at selected trial doses produced significant increases in overall cerebral function, especially in areas associated with the limbic system. Overall, these studies established a semi-quantitative $[^{14}C]$-2-deoxyglucose autoradiographic model in mice, validated it with known pharmacological compounds, and proved its potential usefulness in investigating the central actions of the Ampakines Org 26576 and Org 24448.

The central actions of the Ampakines Org 26576 and Org 24448 were fully and successfully investigated in the murine CNS using the previously established semi-quantitative $[^{14}C]$-2-deoxyglucose model. Acute administration of both Org 26576 and Org 24448 produced dose-dependant effects on LCGU, displaying restricted
regional effects at low doses with increasing cerebrum wide activation at higher
doses. The greatest levels of activation were observed in components of the limbic
and sensory systems with specific effects at low doses in the dentate gyrus, CA3,
subiculum, anteroventral and laterodorsal thalamus, the median forebrain bundle,
cingulate and visual (IV) cortices and the cochlear nucleus (minor sensory activation)
with Org 26576 (1 mg/kg); and the raphe nucleus, medial lateral habenula, CA1 and
medial forebrain bundle with Org 24448 (3 mg/kg). Pre-administration of the
AMPA receptor antagonist, NBQX, blocked the effects of these novel Ampakines on
cerebral activation. This study provides the first evidence for the neuroanatomical
basis of action of these compounds and established that the effects they induce are
indeed directly AMPA receptor mediated.

The effects of chronic administration of both Org 26576 and Org 24448 for both 7
and 28 days on cerebral function and structure/signalling were also successfully
investigated in the mouse cerebrum. Both Org 26576 and Org 24448 administered
chronically induced functional cerebral increases in LCGU in the mouse cerebrum,
with activation of brain regions previously witnessed in the acute study and
recruitment of extra regions including several brain stem and forebrain nuclei such as
the ventral tegmental area of Tsai (VTA), nucleus accumbens, and septum. The
effects were rapid in onset and also persistent for the entire 28-day treatment regime.
Contrarily, chronic administration of both Org 26576 and Org 24448 produced no
effects on hippocampal neurogenesis, or associated receptor/signalling proteins,
suggesting that these novel Ampakines elicit their effects possibly in a neurogenetic
independent manner. This study provides the first evidence for the neuroanatomical
basis of action of these compounds when administered chronically, and is of
importance when considering their prospective role as salutary agents, which would
have to be taken over prolonged periods of time.

The kinetic actions of Org 26576 and Org 24448 were successfully investigated
through the utilisation of extracellular field electrophysiology in the CA1 subfield of
acute hippocampal slices. Both compounds produced dose-dependant increases in
fEPSP amplitude without having any effect on the half-width of responses. A stable
reproducible model of LTP was established in disinhibited slices using HFS, and physiological concentrations of both drugs potentiated a stable form of LTP, with Org 26576 producing a greater more stable potentiation. These results provide the first evidence of the ability of Org 26576 and Org 24448 to affect baseline electrophysiological responses in monosynaptic circuits, and enhance the neural correlate of cognition, LTP.

7.2. Key points and therapeutic implications

The data presented in this thesis, not only provide an anatomical basis for the cerebral activation induced by these compounds, but also go some way to help explain their selectivity and action. Ampakines display subunit selectivity and as AMPA receptor stoichiometry itself is so varied across the brain, the compounds are thought to possibly act in a regionally selective manner. There is evidence supporting this postulation. For example, different Ampakines display varying potencies in different areas of the brain (hippocampus vs. thalamus) (Xia et al., 2005), and within the hippocampus Ampakines have differing effects across sub-populations of cells (Xia and Arai, 2005). Data from this thesis also supports this idea, as behaviourally effective doses of Org 26576 and Org 24448 show restricted regional effects on global metabolic activity, and the distinct drugs also induce differing patterns and hierarchies of cerebral activation. The compounds also display a classical dose-response effect in relation to their effect, as opposed to the biarylsulphonamides compounds, conceivably allowing their effects to be titrated to obtain a desired level of cerebral activation. Work in both acute and chronic paradigms in this thesis has also shown that the compounds also display a very rapid onset of effect, which is undiminished in intensity following prolonged administration. Indeed the response even shows a degree of plasticity over time, switching in activation profiles between one and four weeks. The cerebral activation induced both acutely and chronically is also supportive that these compounds may play a role in various neurological, psychiatric or neurocognitive disorders.

Cognition and the enhancement of memory processes are regarded as the classical areas of application for these compounds. Studies in monkeys, rodents and humans
have shown improvements in the retention of memory, and the acceleration of the acquisition of new memories. Cognitive aspects of schizophrenia have also been positively treated (Johnson et al., 1999; Goff et al., 2001), and there is much scope to treat other conditions such as Alzheimer’s and depression, which display a cognitive deficit component, as well as natural age-related memory deficits. Cognitive dearth during sleep deprivation was also restored through the use of Ampakines (see CX717 study below). In this thesis both Org 26576 and Org 24448 displayed the ability to enhance AMPA receptor kinetics promoting LTP, the neural correlate of memory, in a monosynaptic circuit, the first evidence of their ability to do this. In a recent study by Gary Lynch and colleagues, naturally impaired LTP in middle-aged rats was recovered to normal levels by treatment with the Ampakine CX614 (Rex et al., 2005); further proving the ability of Ampakines to have relevant effects in a truly ‘deficient’ functional model. There is however still much to be determined in advance of these compounds being marketed as agents to improve deficits in normal cognitive functioning. Varying Ampakine compounds have contrary effects on the biophysics of the AMPA receptor response, i.e. effects on duration versus amplitude of fEPSPs, and this greatly impacts on their ability to modulate LTP. Further work is needed to understand these diverse modes of actions and their effects on LTP, to allow the future development of the compounds as memory enhancing drugs (Lynch, 2006).

The exact aetiology of the psychosis schizophrenia is still unclear, but as mentioned previously, research has focused upon excess dopaminergic tone as the primary instigator of the adverse effects witnessed in the condition. It is however becoming clearer that excess dopamine alone cannot account for all the manifestations of the condition, especially the cognitive and negative symptoms. In addition to this, many of the current dopamine antagonist treatments in use result in a hypodopaminergia which is in some cases as debilitating as the positive symptoms of the disease. Similarly, depression is now increasingly being examined as a complex failure of possibly multiple neurotransmitter systems, not just serotonin. This commonality between depression and schizophrenia, evident in the brain regions and signalling cascades involved, lends further support to the so-called ‘network’ theory proposed
in Chapter 5. This theory suggests that elaborate brain networks (multiple transmitters) work together to generate complex behaviours, and failures in this system, such as excess dopamine or loss of serotonergic tone in isolated areas, result in disrupted information processing within these neural networks. Ampakines exert their greatest influence in polysynaptic circuitry as opposed to monosynaptic ones (highlighted by their ability to modulate complex networks in both the acute and chronic \([^{14}\text{C}]-2\text{-deoxyglucose studies in relevant areas}\), and as a result can selectively facilitate the assembly of cortical networks needed to respond to the present behavioural demands. In concert with other regulatory networks that are in play, the compounds can therefore differentially regulate conditions to restore normality or boost deficient function in a particular area displaying failure. Deadwyler’s group in 2005 exhibited this in sleep deprived monkeys, in which CX717 increased cortical activity in depressed areas whilst decreasing it in hyperactive regions, ultimately restoring normality (Porrino et al., 2005). AMPA receptor potentiators can also modulate the release of other neurotransmitters, such as serotonin, acetylcholine and dopamine (Ge et al., 1999; Murray et al., 2003), lending further support to this hypothesis.

Compounds such as CX717 are also being clinically trialled in attention-deficit/hyperactivity disorder (ADHD) sufferers and as treatments for sleep deprivation. In an adult ADHD phase IIa clinical trial in March 2006, CX717 was trialled at 200/800 mg twice daily in a crossover study, and was found to significantly reduce hyperactivity and to a lesser extent, inattentiveness. These results were comparable if not better than those obtained with ‘Strattera’, a norepinephrine reuptake inhibitor medication currently marked by Lilly for ADHD. In another phase IIa clinical trial, the effects on sleep deprivation were investigated. CX717 was found to promote wakefulness, and improved performances on test of attention, delayed recall and visual processing, in sleep-deprived individuals. Other groups have also begun to investigate the potential of these potentiators as neuroprotectants. Compounds such as CX614 have been shown to be able to block excitotoxic brain lesions (Dicou et al., 2003); and in a model of Parkinson’s disease
(6-hydroxydopamine lesions) biarylpropylsulfonamides have prevented cell loss and promote growth/repair of existing cells (GAP-43 staining) (O’Neill et al., 2004).

Following the introduction of Ampakines there was controversy about whether therapeutic positive modulation of AMPA receptor function would result in ultimately positive or negative effects. Due to the imperative role glutamate and its receptors play in manifestation of excitotoxicity (see section 1.6.1.) many believed that over stimulation of AMPA receptors with potentiators could result in neurotoxicity/degeneration or epileptic activity due to hyper-excitable cells, as apposed to positive enhancement of AMPAergic synaptic transmission. This concern arose primarily due to the findings that intraventricular injections of cyclothiazide were lethal or caused seizures, and intracerebral injections resulted in necrotic parenchymal lesions (Taylor et al., 1996). Administered in vitro in combination with agonists, cyclothiazide also proved toxic to cultured hippocampal neurones and glia (May and Robison, 1993; David et al., 1996). However cyclothiazide seems to be the exception to the rule. Overt toxicity phenomena such as seizures, tremor and hyperactivity have not been witnessed with BCP, IDRA-21, and CX516 in either rodents or humans via multiple routes of administration with therapeutically relevant doses; and exposure of cultured hippocampal slices to excessively high concentrations of Ampakines (CX516 1-3 mM) does not produce cell death (Lauterborn et al., 2003). In addition the compounds seen to be well tolerated in humans (Ingvar et al., 1997), and in recent phase IIa clinical trials carried out by Cortex Pharmaceuticals with the Ampakine CX717, drug tolerability was good with no serious adverse effects reported: no changes in blood pressure, heart rate or temperature, and no clinically significant ECG changes were observed (Cortex online presentation). The novel Ampakines Org 26576 and Org 24448 were well tolerated by the mice in all the studies outlined in this thesis, with no detrimental side effects observed.

As Ampakines have no agonist action of their own, and only have subtle effects (as witnessed in behaviour) on AMPA receptor function, they merely enhance natural AMPAergic synaptic transmission, instead of resulting in continual, and potentially
toxic, stimulation. It has also been proposed that drugs that effect deactivation preferentially over desensitisation, as is the case with the Ampakines, have reduced potential toxic effects (Lees, 2000).

7.3. Future directions

Despite all we now know about AMPA receptor potentiators, their pharmacology, toxicology, metabolism and effects both in vitro and in vivo, there is still much to explain. How exactly they exert their effects is still altogether unresolved. Do they purely influence receptor kinetics, or is their action due in some part to downstream and secondary signalling cascades? The role of molecules such as BDNF in their mode of action is still controversial and as yet unresolved.

One possible avenue of investigation may be the further use of non-invasive modalities such as functional magnetic resonance imaging (fMRI), to more precisely image the effects of these Ampakines in animal models. In a manner similar to [14C]-2-deoxyglucose imaging, fMRI examines what is known as the BOLD contrast (the innate magnetic difference between oxy-haemoglobin and deoxy-haemoglobin) in the CNS, as a functional correlate of neuronal activity. This technique has already proved useful in investigating the effects of the AMPA receptor potentiation LY404187 in the CNS of rats (Jones et al., 2005), and the results correlated well with the previous [14C]-2-deoxyglucose imaging work carried out in conscious animals (Fowler et al., 2004). The benefit of fMRI, is that it is translatable between preclinical and clinical studies, and as such may be useful in further delineating the function consequences of these Ampakines in man.

Neurones don’t exist in isolation, and it is highly unlikely that Ampakines act upon neurones alone. Astrocytes, for example, provide both physical and functional support for neurones, acting as an ‘energetic couple’, shuttling glucose and lactate between neurones and blood vessels in response to synaptic activity (Pellerin & Magistretti, 2004). They also play a key role in synaptic transmission, preserving fidelity, through the reuptake of glutamate by reuptake-transporters. Intriguingly, a
recent study by Pierre Magistretti’s group, found that CX546 promoted enhancement of function in cortical and hippocampal astrocytes following glutamate exposure (Pellerin & Magistretti, 2005). It has been suggested that boosting the neuroenergetics of astrocytes (increasing the availability of lactate to neurones) may represent a valuable approach to improving both cognition and neuroprotection (Sapolsky, 2003). It remains to be determined to what extent astrocytes and their modification by Ampakines could play in these processes.

As with other promising drug targets the role of ‘vectorisation’ is often postulated as a possible avenue for investigation. Specifically, designing specific AMPA receptor potentiators to selectively target sub-populations of AMPA receptors possessing particular subunit compositions; hopefully therefore, achieving drug delivery in a regionally selective fashion. In support of this for example, a recent study examining the role of AMPA receptors in depression has attributed the effects of chronic antidepressant treatment to an observed higher expression of GluR1 receptor subunits (Tan et al., 2006). By designing potentiators that ‘mimic’ these effects it could be possible to replicate the effects of antidepressant via this distinctly AMPA mechanism. Vectorisation however, is a concept that has been postulated repeatedly over the years for other receptor targets with little tangible success. Indeed, the concept of selective drug targeting would require further in depth analysis and determination of the exact distribution and stoichiometry of AMPA receptors across the entire cerebrum, in addition to the design of new sub-type selective ligands.

There is however much scope for advancement of Ampakines with further electrophysiological investigation (see discussion Chapter 6 section 6.4.3.). Possibly twinned with molecular studies, electrophysiology would help us to better understand how these compounds at a molecular level interact and function with the AMPA receptor. In support of this, in a seminal paper in 2005 Jin and colleagues studied co-crystal structures of the GluR2 ligand-binding core in complex with the Ampakine CX614 and aniracetam, and as a result of this helped resolve, in a molecular sense, how these compounds elicit their biological effects (Jin et al., 2005). Structure-function studies exploiting these new structural insights, could lead to the design of
more potent specific ligands with even greater functional activity. It is worth noting here that with further appreciation of how these compounds function, care needs to be taken to ensure that any new ligands are not too effective; ultimately resulting in over-potentiation, tipping the balance in favour of excitotoxicity (and seizure activity) with a loss of positive functional effects.

Studies in this thesis have look at functional changes induced by the novel Org compounds in normal healthy animals. To obtain a true measure of the compound’s potential therapeutic usefulness they must be trialled in models either relating to disease states or displaying a deficiency of some sort, be it compromised behaviour or cognitive impairment for example. Transgenic mice, such as the GluR1 knockout or ApoE knockout mice, would be ideal in this regard as they both display clear cognitive dearth. A combination of functional imaging coupled with behavioural testing, as established by Magistretti (Ros et al., 2006), could also prove useful in further investigating the ability of Ampakines to modulate function, but also help define the brain areas involved.

7.4. Conclusion

The data presented in this thesis provide the first evidence for the functional activity induced by the novel Org Ampakines Org 26576 and Org 24448 in the mouse cerebrum, and underscore their potential therapeutic relevance in an array of neurological, psychiatric and neurocognitive disorders.
References


AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. *Neuron* **38**, 611-624.


Uzunov, D.P., Zivkovich, I., Pirkle, W.H., Costa, E., Guidotti, A. (1995). Enantiomeric resolution with a new chiral stationary phase of 7-chloro-3-methyl-3,4-


Abstracts & Publications


Appendix: Solutions and recipes

- **Poly-L-lysine Slides**

  Slides were placed in racks and soaked in poly-L-Lysine (0.1% P8920 Sigma, UK) for 5 minutes. Slides were then dried overnight at room temperature or in an oven at 60 °C for 2 hours. The poly-L-Lysine is reusable several times when store in a sealed container at 4 °C (~1000 slides).

- **10x Phosphate Buffer (PB)**

  2.57 g of NaH₂PO₄ and 11.95 g of Na₂HPO₄ were dissolved and made up to 1 L with distilled water. The solution when mixing does not need to be heated. For Phosphate Buffered Saline (PBS) add 86.67 g NaCl and pH to 7.4. Filter for purity.

- **Paraformaldehyde Fixative 4% (PAM)**

  40 g of paraformaldehyde (weighed in the fumehood) was added to 900 ml of 1 x PB which had been heated to 60-65 °C. The temperature was maintained between 60 and 65 °C until the paraformaldehyde dissolved. The solution was allowed to cool, made up to 1 L with PB and filtered for purity.

- **Citric Acid Buffer**

  2.1 g of citric acid was dissolved in 1 L of distilled water. The pH was adjusted to 6.0 by the addition of 1 M NaOH.

- **Acrylamide Gel Solutions** (To make 2 gels for a large Hoefer gel system):

<table>
<thead>
<tr>
<th></th>
<th>Resolving Gel (10 %)</th>
<th>Stacking Gel (4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>28.35 ml</td>
<td>12.2 ml</td>
</tr>
<tr>
<td>1.5mM Tris pH 8.8</td>
<td>17.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5mM Tris pH 6.8</td>
<td>-</td>
<td>5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>700 μl</td>
<td>200 μl</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>23.1 ml</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>350 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>35 μl</td>
<td>20 μl</td>
</tr>
</tbody>
</table>
- **10x SDS-PAGE Running Buffer**

  30 g of Tris-Base, 144 g of glycine, and 10 g of SDS were fully dissolved in and made up to 1 L with distilled water. The final solution was adjusted to pH 8.3.

- **Western Transfer Buffer**

  16.66 g of Tris-Base and 79.2 g of glycine were fully dissolved in and made up to 4.4 L with distilled water. Immediately prior to use 1.1 L of methanol was added to the solution, to give a final volume of 5.5 L.
Abbreviations

aCSF       Artificial cerebrospinal fluid
AMPA       α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR      AMPA receptor
ATP        Adenosine tri-phosphate
BDP (1-BCP) 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine
BDP-5      1-(1,3-benzodioxol-5-ylcarbonyl)-1,2,3,6-tetrahydropyridine
BDNF       Brain-derived neurotrophic factor
BrdU       (+) 5-Bromo-2'-deoxyuridine
BSA        Bovine serum albumin
Ca2+       Calcium ions
cAMP       Cyclic adenosine mono-phosphate
CNS        Central nervous system
CNQX       6-cyano-7-nitroquinoxaline-2,3-dione
CREB       cAMP response element-binding protein
CX516 (BDP-12) 1-(quinoxaline-6-ylcarbonyl)piperidine
CX546      1-(1,4-benzodioxan-6-ylcarbonyl)piperidine
CX614      2H,3H,6aH-pyrrolidino[2″,1″-3′,2’]1,3-oxazino[6′,5′-
           5,4]benzo[e]1,4-dioxan 10-one
DAB        Diaminobenzine
ECL        Enhanced chemiluminescence
EPSC       Excitatory postsynaptic current
EPSP       Excitatory postsynaptic potential
ERK        Extracellular signal-regulated kinase
fEPSP      Field excitatory postsynaptic potential
GRIP1      Glutamate receptor interacting protein 1
HEK        Human embryonic kidney
HFS        High-frequency-stimulation
IDRA-21    7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine
IgG        Immunoglobulin G
IHC        Immunohistochemistry
LCGU       Local cerebral glucose utilisation
LTD        Long-term depression
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>Magnesium ions</td>
</tr>
<tr>
<td>MK-801</td>
<td>Dizocilpine malonate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBQX</td>
<td>6-nitro7-sulfamoylbenzo(f)quinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature embedding medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Post-synaptic density protein 95</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROD</td>
<td>Relative optical density</td>
</tr>
<tr>
<td>SAP97</td>
<td>Synapse-associated protein 97</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone of the dentate gyrus</td>
</tr>
<tr>
<td>SSRI</td>
<td>Serotonin selective reuptake inhibitor</td>
</tr>
<tr>
<td>STP</td>
<td>Short-term potentiation</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>[$^{14}$C]-2-DG</td>
<td>[$^{14}$C]-2-deoxyglucose</td>
</tr>
</tbody>
</table>