AMPA receptor potentiators:

Mechanisms of Neuroplasticity

Oliver Paul Voss B.Sc. (Hons)

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University of Edinburgh

Declaration

This thesis comprises my own work, unless otherwise acknowledged, and has not previously been presented for a degree in any form.

Oliver Paul Voss

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List of Abbreviations

2-APV	2-amino-5-phosphonovaleric acid
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine
6-OHDA	6-hydroxydopamine
AD	Alzheimer's Diesease
ADHD	Attention Deficit Hyperactivity Disorder
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
s-AMPA	S-enantomer and active form of AMPA
ANOVA	Analysis of Variance
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
AP	Alkaline Phosphatase
Bcl-2	B-Cell Lymphoma Protein 2
BDNF	Brain Derived Neurotrophic factor
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
CaMKII	Calcium Calmodulin Kinase II
Cdk5	cyclin-dependent kinase 5
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central Nervous System
CREB	cAMP Response Element Binding Protein
DA	Dopamine
DAB	Diaminobenzine

DG	Dentate Gyrus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNQX	6,7-Dinitroquinoxaline-2,3-dione
DOPAC	dihydroxyphenylacetic acid
ECL	Entorhinal Cortex Lesion
ECM	Extra-Cellular Matrix
ECS	Electro Convulsive Seizures
ECT	Electro Shock Therapy
EDTA	ethylene-diamine-tetra-acetic acid
EGF	Epidermal Growth Factor
ERK 1/2	Extra-cellular Regulated Kinase 1/2
MAPK 44/42	Mitogen Activated Protein Kinase 44/42
FAK	Focal Adhesion Kinase
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
GDEE	Glutamic acid diethylester
GEF	Glutathione Exchange factor
GluR1-4	Glutamate Receptor sub-units 1-4
GTP	Guanosine tri-phosphate
HCL	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HVA	Homovalinic acid
IGF	Insulin derived growth factor

IgG	Immunoglobulin G
iGluR	Ionotropic Glutamate Receptors
KA	Kainic acid (Kainate)
LTD	Long Term Depression
LTP	Long Term Potentiation
MAO	Monoamine oxidase
MAPK	Mitogen Activated Protein Kinases
MCI	Mild Cognitive Impairment
mGluR	Metabotrophic Glutamate Receptors
MK-801	Dizocilpine,
mRNA	Messenger Ribonucleuic Acid
NA	Noradrenaline
NF-H	Neurofilament-Heavy Chain
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartic acid
NP40	Nodipet 40
NSF	N-ethylaminde sensitive factor
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
OD	Optical Density
PB	Phosphate Buffer
PBS	Phosphate Buffered Saline
PCA	Perchloric Acid
PCP	Phencyclidine

PD	Parkinson's Disease
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PI(3)K	Phosphoinositide 3-kinases
PNS	Peripheral Nervous System
PTSD	Post-Traumatic Stress Disorder
PVDF	Polyvinylidenefluoride
RA	Retinoic acid
SD	Standard Deviation
SEM	Standard Error of the Means
SSRI	Selective Serotonin Re-Uptake Inhibitors
SVZ	Sub-ventricular Zone
SGL	Sub-Granular Layer
TARP	Transmembrane AMPA Receptor Binding Proteins
TGF-β	Transforming Growth Factor beta
TH	Tyrosine Hydroxylase
VGCC	Voltage Gated Calcium Channels

Summary

The majority of synaptic transmission occurring at mammalian central synapses is mediated by glutamate acting on specific trans-membrane receptors. The discovery that certain forms of synaptic plasticity believed to underlie different forms of learning and memory were mediated by glutamate receptors led to the development of a series of compounds that could facilitate this process, through the positive modulation of AMPA receptors. These compounds, termed collectively AMPA potentiators, were originally developed as cognitive enhancers but AMPA potentiators have also been shown to enhance the expression of the neurotrophin BDNF. This has led to the prediction that AMPA potentiation may be able to enhance cellular resilience and survival, stimulate neurogenesis and promote synaptic integrity. In this thesis two principal aspects of AMPA potentiation were addressed: their ability to induce structural plasticity *in vitro* and *in vivo* and their ability to produce long-lasting, persistent changes in brain biochemistry and neuronal development.

The effect of an AMPA potentiator on structural plasticity *in vitro*

The human neuroblastoma cell line SH-SY5Y was used as a model for *in vitro* neuroplasticity. A statistically robust and reproducible method for measuring the average length of neuritic processes was developed from first principles. The effects on neurite growth of the AMPA potentiator LY404187 were investigated using this method. LY404187 (0.1-1μM) significantly increased the average length of neurites only in the presence of the receptor agonist s-AMPA (10μM) and was dependent on AMPA receptor activation. LY404187 also increased expression of the cytoskeletal protein

neurofilament–heavy chain. LY404187 increased neurite length in a BDNF-dependent manner as co-incubation of SH-SY5Ys with an antibody specific to BDNF blocked the increase in neurite length.

The effect of an AMPA potentiator on structural plasticity *in vitro*

The ability of LY404187 to enhance structural plasticity was tested in *in vivo*. Unilateral excitotoxic lesions of the entorhinal cortex in mice led to a loss of synapses in the ipsilateral dentate gyrus. In order to establish whether LY404187 could promote the regeneration on synapses in this model (0.5mg/kg s.c) was administered twice daily for 14 and 28 days. Immuno-histochemical analysis revealed that although the lesion was successful in producing a significant ipsilateral loss in synapse density, LY404187 administration had no significant effect on structural plasticity.

The rate of neurogenesis in the dentate gyrus following lesioning and LY404187 administration was also measured. Immuno-histochemical detection of newly generated cells within the subgranular layer of the dentate gyrus revealed no significant change in the number of cells in either hemisphere 14 and 28 days following ECL with or without administration of LY404187 (0.5mg/kg s.c).

Biochemical changes *in vivo* associated with chronic AMPA potentiation

The ability of the AMPA potentiator LY450108 (0.5mg/kg s.c.) was tested for its ability to induce long-lasting changes in mouse brain biochemistry and physiology. Using a dosing paradigm aimed at identifying the chronic and long lasting effects of

AMPA potentiation *in vivo* three studies were carried out to investigate multiple end points: protein expression down stream of AMPA receptor potentiation, monoamine neurotransmitter levels and hippocampal neurogenesis.

Two potential effectors of AMPA potentiator-mediated neuroplasticity are ERK1/2 (MAP 44/42) and the transcription factor CREB. Using western blotting and immunohistochemistry techniques, phosphorylation levels of these key proteins were monitored in the hippocampus. LY450108 produced no significant change in the levels of pERK1/2 in the whole hippocampus when measured by Western Blotting. Immuno-histochemical detection of pERK1/2 and pCREB in individual cells of the denate gyrus, CA1, CA2 and CA3 allowed for a more quantative assessment of protein phosphorylation following AMPA potentiation. Statistical analysis showed that LY450108 produced no significant changes in protein levels across all regions and time points.

Adult neurogenesis is believed to be a crucial mediator of certain forms of cognitive and behavioural plasticity. Recently it in the actions of common antidepressants and forms a key area of the neurotrophin hypothesis of depression. AMPA potentiators increase the expression of neurotrophins in the hippocampus where they may regulate cell survival and development. AMPA potentiators have been shown to increase the rate of neurogenesis in the hippocampus following chronic and acute administration. Using BrdU as a marker for newly generated cells and NeuN as a neuronal marker the rate of neurogenesis and neuronal development was measured. Statistical analysis of $BrdU^+$ cells in the subgranular layer of the hippocampus revealed no significant change following administration of LY450108 across all time points. The development of newly generated cells can be assessed by double labelling of $BrdU^+$ cells

with other cell-type specific markers. The percentage of $BrdU^+$ cells that were also positive for the neuronal marker NeuN was used as an assessment of the rate of neuronal development in the dentate gyrus. Statistical analysis revealed no significant change in the percentage of double labelled cells following administration of LY450108 across all time points.

AMPA potentiators may have therapeutic potential in the treatment of depression. They have been shown to be active in animal models of depression. They also act as cognitive enhancers and have been shown to enhance BDNF expression and neurogenesis, both of which have recently been implicated in depression and the mechanisms of action of common antidepressants. All of the most commonly prescribed antidepressants increase synaptic levels of monoamine neurotransmitters. In this study the effect of chronic administration of LY450108 on the levels of NA, 5HT and DA and their principal metabolites was investigated. High Performance Liquid Chromatography allows for rapid resolution of the components of biological mixtures. Electrochemical detection allows for highly sensitive (picogram) quantification of the concentration of each component in real time. The levels of monoamines detected in these experiments were consistent with published studies. Statistical analysis revealed no significant change in the levels of any of the monoamines measured following administration of LY450108.

AMPA potentiaton holds promise for the treatment for many CNS disorders. This thesis provides support for this hypothesis by demonstrating that AMPA potentiation can induce structural plasticity in a BDNF dependant mechanism. Although the *in vivo* studies were inconclusive the consequences of chronic AMPA potentiator administration are important and merit further investigation.

1. Introduction

1.1 AMPA receptors

1.1.1 Glutamate as a neurotransmitter

Although evidence for a role of glutamate in neurotransmission first appeared over 50 years ago (Hayashi 1954) it was not until 20 years later, when significant pharmacological, biochemical and immuno-histochemical evidence had been accumulated, that glutamate became widely accepted as the primary excitatory neurotransmitter in the mammalian brain (reviewed in Watkins 2000). Early experiments carried out by Curtis and Watkins on the excitatory effect of exogenously applied glutamate on cultured spinal neurons (Curtis, Phillis et al. 1959a; Curtis, Phillis et al. 1959b) and subsequent structure-function studies, led to the "3-point receptor" model describing the conserved structural requirements of different glutamate analogues (Watkins 2000). However, the abundance of glutamate in the human brain, its role in intermediary metabolism and protein synthesis, coupled with a lack of specific antagonists for glutamate-induced excitation, delayed the acceptance of glutamate as a neurotransmitter. The earliest evidence for the existence of multiple glutamate receptors came from studies showing heterogeneous responses across different thalamic and spinal nuclei to glutamate analogues (McLennan, Huffman et al. 1968; Duggan 1974; McCulloch, Johnston et al. 1974). However these data were interpreted as showing glutamate acting on different populations of excitatory synapses rather than through different receptor subtypes. Glutamate receptor pharmacology during the late 1970s, particularly in the UK, led to the synthesis and discovery of over 75 compounds with

excitant properties similar to glutamate (for a review seeMcLennan 1983). Among these, NMDA (Curtis and Watkins 1960), and the naturally occurring kainate and quisqualate (Shinozaki and Konishi 1970; Shinozaki and Shibuya 1974), were the most potent and had the most distinct excitatory profiles. However it was not until the discovery of selective antagonists to these compounds were discovered, that the notion of glutamate receptors involved in neurotransmission began to be more widely accepted. The first conclusive evidence for the existence of multiple receptor subtypes came from studies on the effects of Mg^{2+} and D-α-aminoadipate (DαAA) on NMDA-induced excitation (Biscoe, Evans et al. 1977; Evans, Francis et al. 1978). This led to the classification of glutamate receptors into three main groups, determined by their response to various antagonists. Thus NMDA and L-aspartate responses were sensitive to Mg^{2+} and DαAA, whereas both quisqualate and l-glutamate-induced excitation was sensitive to L-glutamic acid diethylester (GDEE) with kainate induced responses being insensitive to either antagonist. This classification of NMDA, quisqualate and kainate preferring receptors (Watkins and Evans 1981) has remained relevant to this day and is a testament to the quality of two decades of pharmacological and electrophysiological investigation.

Throughout the 1980s, a range of more specific pharmacological tools was developed: the NMDA receptor antagonists, 2-amino-5-phosphono-valerate (2APV) (Davies, Francis et al. 1981), MK-801 (Wong, Kemp et al. 1986) and PCP (Lodge and Anis 1982), the highly selective agonist for GDEE sensitive receptors alpha-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid (AMPA) (Krogsgaard-Larsen, Honore et al. 1980) and the selective antagonist CNQX (Honore, Davies et al. 1988). These

developments supported the existent classification system and confirmed glutamate receptors as the mediators of synaptic transmission in the central nervous system. Advances in radioligand binding studies (Monaghan and Cotman 1982; Monaghan, Yao et al. 1984; Olverman, Jones et al. 1984) and immuno-histochemical techniques (reviewed in Iversen 1978 and Cuello 1978), were crucial to the acceptance of the existence of multiple glutamate receptors involved in synaptic transmission.

As well as the established NMDA, quisqualate and kainate receptors, the existence of another class of receptor, insensitive to NMDA and non-NMDA antagonists, was being documented, (McLennan 1983). The observation that glutamate was capable of inducing second messenger molecules, and mobilising intracellular Ca^{2+} and the cloning of a glutamate receptor with distinct biochemical properties (Sladeczek, Pin et al. 1985; Nicoletti, Iadarola et al. 1986; Nicoletti, Meek et al. 1986; Sugiyama, Ito et al. 1987) opened up the field of metabotropic glutamate receptor research.

During the early 1990s a number of groups began cloning and expressing functional recombinant glutamate receptors in mammalian cells, this led not only to the elucidation of their structure but also to the reclassification of glutamate receptors by their molecular, rather than pharmacological, properties (Figure 1.1). cDNA encoding a glutamate receptor similar to frog and chick kainate binding proteins designated GluR-K1 (later shown to be GluRA) was first cloned by Hollmann and colleagues (Hollmann, O'Shea-Greenfield et al. 1989). In 1990 three groups reported the cloning of functional AMPA-type glutamate receptors (Keinanen, Wisden et al. 1990; Nakanishi, Shneider et al. 1990; Sakimura, Bujo et al. 1990), but it was the work of Seeburg and colleagues that was most comprehensive in its characterisation of the AMPA receptor subunits. They

cloned four cDNAs that encoded proteins of approximately 900 amino-acids in length and shared between 56% and 70% sequence homology (Keinanen, Wisden et al. 1990). The patterns of mRNA expression in rat brain and their pharmacology when expressed in Xenopus oocytes, indicated that they had cloned a family of AMPA-type glutamate receptors. In the following years a rush to clone other glutamate receptors lead to the generation of recombinant NMDA receptors (Moriyoshi, Masu et al. 1991), kainate receptors (Werner, Voigt et al. 1991) and metabotropic glutamate receptors (Houamed, Kuijper et al. 1991; Masu, Tanabe et al. 1991). Since the creation of recombinant receptors the detailed molecular structure and the function of glutamate receptors has been intensively studied (Hollmann and Heinemann 1994; Dingledine, Borges et al. 1999). Enormous advances have been made in the characterisation of the structure and function of AMPA receptor subunits from the structural basis of calcium permeability (Verdoorn, Burnashev et al. 1991), glutamate binding site structure (Armstrong, Sun et al. 1998), mechanisms of receptor desensitisation (Sun, Olson et al. 2002) and conformational states of native receptors (Nakagawa, Cheng et al. 2005).

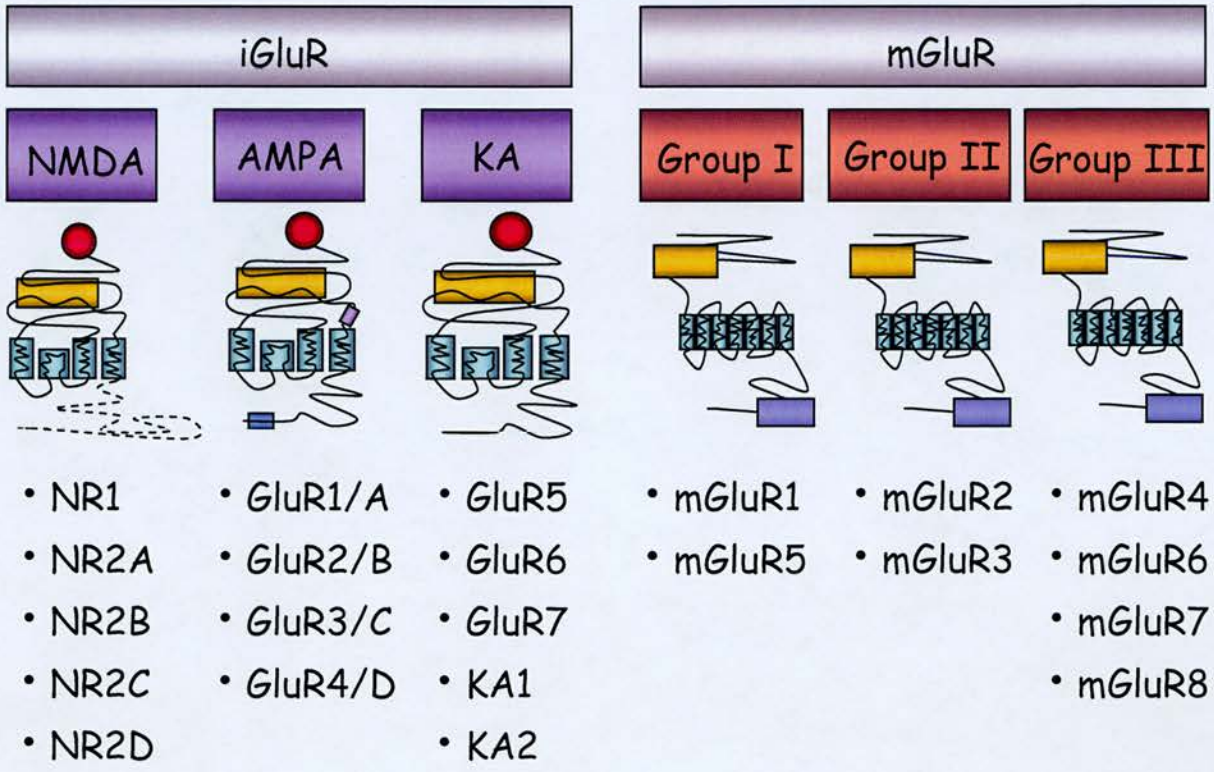


Figure 1.1 Glutamate receptor classification

Glutamate receptor subtypes based on molecular classification. Receptors are divided into metabotropic and ionotropic receptors depending on their permeability to ions or their ability to activate second messenger system. There are three classes of ionotropic receptors: NMDA (composed of NR1 and NR2A-D subunits), AMPA (composed of GluRA-D/GluR1-4 subunits) and Kainate (composed of GluR5-7 and KA1-2 subunits). All ionotropic receptor subunits have an external N terminal, 4 trans-membrane domains, a re-entrant loop between TM 2/3 and an internal C-terminus. Subunits assemble into homo- or hetero- tetramers. Adapted from Dingledine (1999).

1.1.2 AMPA receptor structure

Intense research following the cloning of AMPA receptors in 1990 led, in a short space of time, to the characterisation of a number of major structural features of AMPA receptor subunits that determined the biophysical properties of the associated ion channels. Most of these features are either slice variants or RNA editing sites. The first such feature was the so-called Flip/Flop site, a 38 amino acid sequence preceding the fourth transmembrane region (IV) (Figure 1.2). Alternative splicing of the gene during development changes the composition of this area by 9-11 residues, determining the desensitisation dynamics of the receptor. Seeburg and colleagues showed that flip form containing receptors desensitised less rapidly than flop forms, allowing greater ion flow through the receptor (Sommer, Keinanen et al. 1990). Interestingly they proposed that this form of alternative splicing may underlie some forms of synaptic plasticity. Studies into the current-voltage relationship of recombinant receptors revealed that the GluRB subunit had unique rectification properties and concluded, through site directed mutagenesis studies, that the identity of the residue at position 586, located in the second transmembrane region (II) determined the calcium flow through the receptors (Hume, Dingledine et al. 1991; Verdoorn, Burnashev et al. 1991). GluRA, C and D subunits all have a neutral glutamine (Q) residue at that point, whereas GluRB subunits have a positively charged arganine (R). This difference between subunits was shown to be a result of RNA editing following transcription of a glutamine encoding transcript (Sommer, Kohler et al. 1991). It was also shown that most native receptors behaved like GluRB containing recombinant heteromers although there were notable exceptions, in a

subset of neurons in the hippocampus, retinal bipolar cells and Bergman Glia in the cerebellum (Nakanishi 1992) indicating the important role GluRB subunits play in *in vivo* synaptic transmission.

C-terminal splice variants add further variety to AMPA receptor structure. GluRB and D display alternative splice forms that contain a longer and shorter C terminal region respectively (Gallo, Upson et al. 1992; Kohler, Kornau et al. 1994). Although the exact functional implications of this variability have not been elucidated, GluR4c has been shown to be widely expressed in the human brain (Kawahara, Ito et al. 2004) and interactions between the C terminal region of GluRB subunit and intracellular proteins, through the PDZ binding motif, have been implicated in the establishment and expression of certain forms of synaptic plasticity (Collingridge and Isaac 2003; Malenka 2003). The first crystal structure of the glutamate binding site of AMPA receptors was provided by Armstrong et al. (1998) and led in the ensuing years to greater understanding of the mechanics of channel activation and the action of agonist and partial agonists. In an elegant series of experiments, researchers in the lab of Gouaux created a glutamate binding core comprising the S1 and S2 domains of GluR2 linked by a hydrophilic linker (Armstrong, Sun et al. 1998). The structure of this chimera bound to kainate was resolved revealing a clam-shaped structure with glutamate bound at the interface of the two regions. The kainate bound formation was shown to be at an intermediate closure state between that of the glutamate-binding protein (QBP) open and closed conformations. This and subsequent experiments with a range of antagonists and full and partial agonists (Armstrong and Gouaux 2000) showed that the S1S2 domain closed to different degrees depending on the bound ligand; KA produced a closure of

12° whilst AMPA caused a shift in the domains of around 20°. They concluded from their experiments that the extent to which the channel is opened depends on the extent of the domain closure (Armstrong and Gouaux 2000; Erreger, Chen et al. 2004). The competitive antagonist DNQX produced no change indicating that antagonists may stabilize the binding region in the open conformation preventing any channel opening. This work was carried forward by Jin et al which showed a correlation between the potency of five agonists, 5-substituted willardines; a series of partial agonists that each differed by just one atom, and the extent of domain closure of the ligand binding core (Jin, Banke et al. 2003).

AMPA receptors are characterized by very rapid (millisecond) desensitisation (Hollmann and Heinemann 1994; Jones and Westbrook 1996; Dingledine, Borges et al. 1999). Although this process had been very well defined using electrophysiological techniques, it was not until recently that a mechanistic model explaining this phenomenon was proposed. Sun et al. (2002) proposed that AMPA receptors behave as a 'dimer of dimers' whereby the S1 region of the glutamate binding regions of two subunits interact regulating the opening and desensitisation of the channel. The researchers proposed that following agonist binding the binding region closed whilst the SI regions remained stable, transferring the strain associated with this shift to the transmembrane channel-forming region of the subunits leading to the channel opening. Following this there is a rearrangement of the dimer interface leading to a stable desensitised state in which glutamate binding is no longer coupled to channel opening. The agonist remains bound, the channel remains closed and the receptor remains insensitive to glutamate (Sun, Olson et al. 2002; Erreger, Chen et al. 2004). This period

of deactivation and desensitisation following receptor activation shapes the post synaptic response to presynaptic glutamate release (Jones and Westbrook 1996). This feature of AMPA receptors is not fixed. Structural features of GluRA-D subunits, such as the flip/flop region, change the desensitisation dynamics of the receptor (Sommer, Keinanen et al. 1990) and the auxiliary AMPA subunit Stargazin (see below) blocks desensitisation and increases AMPA mediated currents *in vivo* (Yamazaki, Ohno-Shosaku et al. 2004; Priel, Kolleker et al. 2005; Tomita, Adesnik et al. 2005). The ability of a receptor to alter its response to agonist binding depending on intrinsic (splice variation, auxiliary subunits) or extrinsic (strength and duration of synaptic activity) factors adds further evidence to the notion of a highly plastic and dynamic postsynaptic complex.

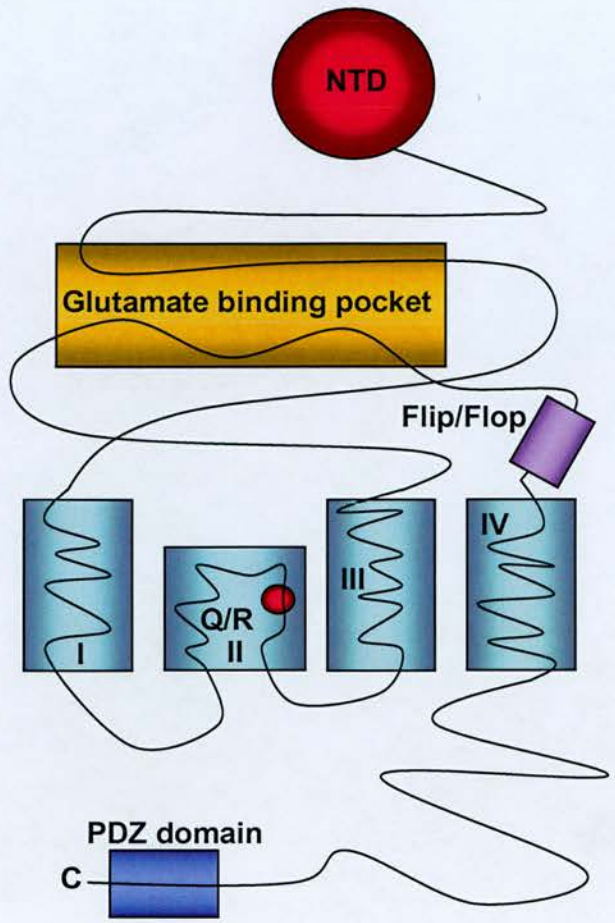


Figure 1.2 Structure of the AMPA receptor GluR2/B subunit

Illustration showing the major structural features that regulate the receptor's activation and desensitisation dynamics as well as its interaction with intracellular proteins that govern receptor localisation and trafficking: a large extra-cellular N-terminal domain, the clam shaped glutamate binding domain, four transmembrane domains (I-IV) and an intra-cellular C-terminal domain, containing PDZ interacting domains. Key determinants of ion channel properties: Flip/Flop region and Q/R splice site. The glutamate binding region and the principal region involved in protein-protein interaction. Other AMPA subunits contain all these features and share between 56% and 70% sequence homology.

1.1.3 AMPA receptor mediated synaptic plasticity

Research in the field of behavioural pharmacology has gone a long way over the past 40 years to answer some of the fundamental questions surrounding the mechanism underlying complex behaviour (Robbins and Murphy 2006). Elucidating the mechanisms of synaptic plasticity, first postulated by Donald Hebb as being the main substrate of memory formation (Hebb 1949), has dominated the research in this field. As the role of glutamate as a neurotransmitter was being revealed and the pharmacological tools became available to study its actions, the phenomenon of Long Term Potentiation (LTP) and its role in memory formation became apparent (Collingridge and Bliss 1995).

Synaptic plasticity is a highly complex field and has been reviewed in detail elsewhere (Malenka 2003; Lynch 2004; Malenka and Bear 2004). There are, however, a number of key features that are relevant to this thesis: The first is the fundamental mechanism by which LTP is initiated. Repeated activation of AMPA receptors depolarises the postsynaptic membrane that in turn releases the voltage dependent magnesium block of NMDA receptors that gate the flow of Ca^{2+} into the cell. The rise in intracellular calcium has a number of downstream effects, mediated in part by CaMKII and CREB, that trigger the expression of LTP, partly through the process of synaptic tagging (Frey and Morris 1997; Frey and Morris 1998; Henley 2001; Bredt and Nicoll 2003; Malenka 2003; Cavazzini, Bliss et al. 2005). Key observations that helped develop this model have shown that blockade of NMDA receptors suppresses LTP formation *in vitro* (Collingridge, Kehl et al. 1983) and impaired certain forms of

memory *in vivo* (Morris, Anderson et al. 1986). Second is the principal expression of a potentiated synapse; the modification of AMPA receptor stoichiometry, density and phosphorylation (Song and Huganir 2002). Proteins such as NSF (Dong, Zhang et al. 1999), GRIP (Dong, O'Brien et al. 1997; Dong, Zhang et al. 1999) and PICK1 (Xia, Zhang et al. 1999) have all been shown to regulate the surface clustering of AMPA receptors through interaction with the C terminus of GluR subunits and are involved in the expression of LTP (Henley 2001; Malenka 2003). Evidence suggests that these interactions may be transient and work in concert with other scaffolding proteins to regulate AMPA subunit trafficking (Fukata, Tzingounis et al. 2005). Over the last few years a family of proteins that have a much more intimate association with AMPA receptors and regulate their localisation and their pharmacological properties has been characterised. Stargazin or γ-2 was first cloned as a putative calcium channel γ-subunit that was absent in mice carrying a spontaneous mutation, so called 'stargazer' mice (Letts, Felix et al. 1998). These mice developed spontaneous seizures that led to their characteristic rearing that gave them their name. Investigations into the development of cerebellar granule cells revealed that stargazer mutant mice displayed synaptic responses lacking an AMPA receptor mediated component (Hashimoto, Fukaya et al. 1999). This observation was investigated further by Chen and colleagues who made the important observation that stargazin interacts with AMPA receptors and regulates their synaptic targeting (Chen, Chetkovich et al. 2000). They also demonstrated that stargazin did not alter the calcium currents in the cell, concluding that the primary role of the protein was to regulate AMPA receptor trafficking, rather than to act as a calcium channel (Chen, Chetkovich et al. 2000). A family of stargazin-like proteins named γ3, 4 and 8, that bind

with AMPA receptors, were soon identified and named transmembrane AMPA receptor regulatory proteins (TARPs). Recently TARPs have been shown to enhance AMPA mediated currents *in vivo* (Yamazaki, Ohno-Shosaku et al. 2004) by reducing desensitisation, accelerating recovery from desensitisation and slowing deactivation (Priel, Kolleker et al. 2005; Tomita, Adesnik et al. 2005; Turetsky, Garringer et al. 2005). Evidence on the role of stargazin in regulating AMPA *in vivo* suggests that potentiation of AMPA receptors may enhance physiological mechanisms (Nicoll, Tomita et al. 2006; Tomita, Sekiguchi et al. 2006)

The receptor composition is also important to the expression of LTP, with the synaptic delivery of GluR1/2 subunits a key step in this process. (Shi, Hayashi et al. 2001). The discovery of 'silent' synapses provided strong evidence for the role of AMPA receptors in the expression of LTP. These synapses are devoid of synaptic AMPA receptors but have NMDA receptors. LTP can be induced at these synapses by the delivery of AMPA receptors to the synapse (Gomperts, Rao et al. 1998). The phosphorylation of AMPA receptors during LTP has also been shown to modulate their function (Benke, Luthi et al. 1998; Nicoll and Malenka 1999). These two aspects are relevant to this thesis because AMPA potentiation can be hypothesised to induce the induction of LTP and mimic or amplify aspects of its expression. Both these processes might be important in providing cognitive enhancement.

1.1.4 AMPA receptors and cognitive function

Following the seminal observation that NMDA receptors were essential for the formation of hippocampal dependant memories (Morris, Anderson et al. 1986) a number of studies showed that these mechanisms were also important in the formation of fear conditioning in the amygdala (Miserendino, Sananes et al. 1990), stimulus-response learning in the striatum (Packard and Teather 1997) and the acquisition of approach behaviour in the nucleus accumbens (Di Ciano, Cardinal et al. 2001). Recently it has become clear that although NMDA receptor function is crucial for the formation of behavioural responses and memories, AMPA receptors are crucial for the formation and retrieval of certain forms of memory (Day, Langston et al. 2003; Bast, da Silva et al. 2005). Thus blockade of AMPA receptors disrupts recall as well as encoding (Robbins and Murphy 2006). The use of pharmacological intervention in behavioural tests using rats has been paralleled by genetic studies using mice (Reisel, Bannerman et al. 2005; Schmitt, Sprengel et al. 2005) and researchers have been able to dissect specific forms of memory formation (Bannerman, Rawlins et al. 2006).

As pharmacological and genetic studies prove, disruption of AMPA and NMDA receptors has profound effects on performance in certain established behavioural paradigms. Synaptic disruption in humans is obviously less amenable to intervention. However there are a number of key observations that show that the mechanisms involved in memory and behaviour in humans are broadly similar to those seen in experimental animals. In conditions such as Alzheimer's disease, that are characterised by widespread and progressive loss of synapses in cortical and limbic areas, the loss of

long term memories and cognitive functions are the most striking symptoms. The most convincing evidence comes from patients who have taken drugs that specifically interfere with glutamate receptor function. The compounds PCP and ketamine, which both suppress the formation of LTP through their antagonism of NMDA receptors (Stringer, Greenfield et al. 1983; Zhang and Levy 1992), also impair performance in memory tests in humans that are comparable to conventional animal tests (Honey, Honey et al. 2005; Rowland, Astur et al. 2005). At higher doses these compounds produce profound schizophrenia-like symptoms that have been exploited to study the mechanisms of this condition in animal models (Morris, Cochran et al. 2005).

In the brain 60% of all neurons, including all cortical pyramidal neurons and thalamic relay neurons utilise glutamate as their primary neurotransmitter (Javitt 2004). Modulation of glutamatergic synaptic transmission is involved in higher cognitive processes many of which are severely affected in neuropsychiatric conditions such as depression and schizophrenia. Neurodegenerative conditions such as Alzheimer's and Parkinson's diseases are also characterised by the loss of synapses in cortical and sub-cortical areas. Synaptic transmission through glutamate receptors has therefore become a popular target for therapeutic intervention. AMPA potentiators are currently in clinical trials for the treatment of depression, anxiety, schizophrenia, mild cognitive impairment, ADHD, Alzheimer's and Parkinson's disease. For each of these conditions there is substantial evidence for impairment of glutamatergic transmission.

The modulation of synaptic transmission through glutamate receptors is critical for the best studied form of synaptic plasticity, LTP. Most neuroscientists now believe this process to be a fundamental substrate for learning and memory. Over the last

decade there has been a parallel effort to synthesise compounds that can alter this process with the view of creating viable clinical compounds for the treatment of age-related and pathological cognitive disorders.

1.2 AMPA receptor potentiators

1.2.1 Introduction

The role of glutamate receptors in the formation and expression of synaptic plasticity and consequently in higher cognitive function provided the intellectual basis for the development of positive modulators of AMPA receptors (Morris, Anderson et al. 1986; Lynch, Muller et al. 1988; Muller, Joly et al. 1988; Muller and Lynch 1988). The central hypothesis predicts that positive modulation of AMPA receptors will facilitate the induction of LTP by lowering the threshold for NMDA receptor recruitment. This would lead to cognitive enhancement, with obvious clinical benefit (Lynch 2004). During the early 1990s a number of compounds were shown to be capable of potentiating AMPA receptor mediated currents. The plant lectin concanavalin-A was the first compound shown to act as a positive modulator of quisqualate receptors at mammalian synapses (Mayer and Vyklicky 1989). Soon after, the nootropic agents aniracetam and piracetam were identified as AMPA potentiators (Ito, Tanabe et al. 1990), able to reverse chemically-induced memory impairment in rats (Lazarova-Bakarova and Genkova-Papasova 1989) and improve memory in humans (Lee and Benfield 1994). Finally the diuretic diazoxide, and particularly its derivative cyclothiazide, was shown to potentiate AMPA receptor responses with much higher potency than aniracetam (Yamada and Rothman 1992; Yamada and Tang 1993).

1.2.2 Classes of AMPA receptor potentiator

These discoveries led to the synthesis of the principal classes of AMPA potentiator. Gary Lynch and colleagues developed a series of aniracetam derivatives, bezoylpiperidines, termed 'AMPAkines'. The prototypical AMPAkine, CX-516, was shown to potentiate synaptic transmission *in vitro* with much higher potency then previously described compounds. Crucially it could facilitate the induction of LTP (Arai, Guidotti et al. 1996), cross the blood-brain barrier, potentiate central transmission and facilitate memory formation with much higher potency then previously described compounds such as aniracetam and piracetam (Staubli, Perez et al. 1994; Staubli, Rogers et al. 1994).

The thiazide derivative cyclothiazide is the most well-characterised AMPA potentiator and remains an important tool to investigate the mechanisms of AMPA potentiation (Sun, Olson et al. 2002). It is not, however, clinically relevant due to its poor brain penetration. However, IDRA-21, another thiazide, has been shown to potentiate AMPA mediated transmission *in vitro* and *in vivo* (Bertolino, Baraldi et al. 1993; Zivkovic, Thompson et al. 1995)

The final major class of AMPA potentiator are the biarylpropylsulphonamides. These were first synthesised by Eli Lilly (Ornstein, Zimmerman et al. 2000) and have been shown to be the most potent AMPA potentiators so far described. They are highly selective potentiators of AMPA receptors and show good brain penetration and are active in a number of animal behavioural models (Bai, Li et al. 2001; Baumbarger,

Muhlhauser et al. 2001; Li, Tizzano et al. 2001; Vandergriff, Huff et al. 2001; Quirk and Nisenbaum 2002).

Recently Lilly Neuroscience have described two further generations of AMPA potentiator that have nanomolar potencies at recombinant AMPA receptor subunits expressed in *Xenopus* oocytes (Shepherd, Aikins et al. 2002; Zarrinmayeh, Tromiczak et al. 2006).

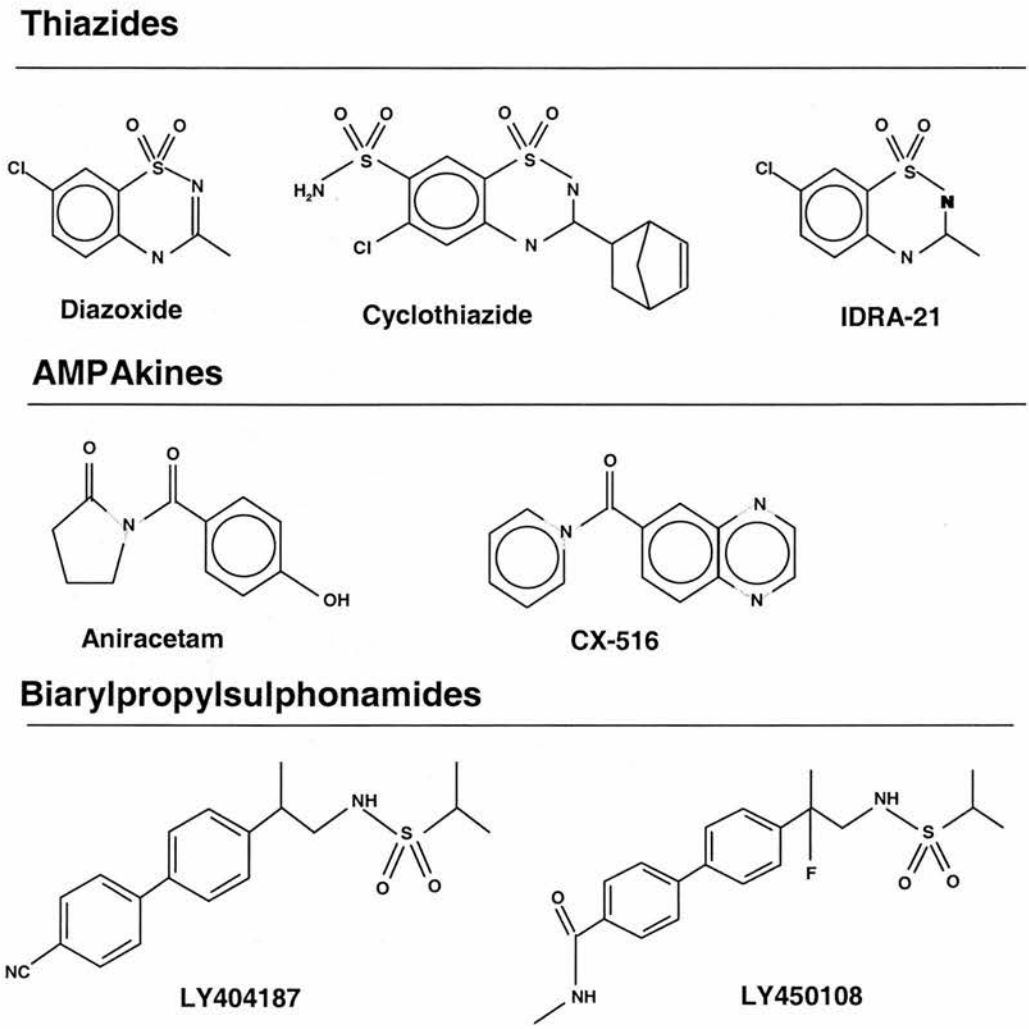


Figure 1.3 Classes of AMPA potentiators

Chemical structures of representative members of the principal classes of positive AMPA receptor modulators. (Kessler and Arai 2006)

1.2.3 Pharmacology of AMPA receptor potentiators

Positive allosteric modulators of AMPA potentiators of different chemical classes display a range of potency and efficacy. However, they share a number of key features that give them their basic properties. Firstly they are unable, on their own, to induce AMPA receptor activation. Secondly, upon binding of the agonist, the period of channel opening is prolonged and the amplitude of current is increased (Figure 1.4). Where they differ is in their binding regions and consequently, their mechanisms of receptor potentiation. The exact nature of the binding site for allosteric modulators remains uncertain. A number of studies have shown that AMPA potentiators show selectivity between flip and flop variants of receptor subunits (Partin, Bowie et al. 1995; Arai, Xia et al. 2002; Quirk and Nisenbaum 2003) and site mutagenesis has revealed that the potencies of AMPA modulators are particularly sensitive to changes in the flip-flop region. However the exact mechanisms of action appear to be distinct between different classes of compound. AMPAkines appear to be more selective for flop over flip splice variants (Kessler and Arai 2006), while biarylpropylsulphonamide compounds and benzothiazides are more selective for flip over flop splice variants (Ornstein, Zimmerman et al. 2000; Gates, Ogden et al. 2001; Miu, Jarvie et al. 2001). There are also class differences between selective effects on the deactivation and desensitisation of AMPA receptors. Aniracetam has been shown to slow desensitisation of AMPA receptors, prolonging the opening time for the channel and increasing the peak current flow (Isaacson and Nicoll 1991). Aniracetam is also more selective for flop variants of GluR subunits (Xiao, Staubli et al. 1991). AMPAkines, although structurally related to

aniracetam, have been shown to separate into two subfamilies that differ in their respective actions (Arai, Kessler et al. 1996; Arai, Kessler et al. 2000; Arai, Xia et al. 2002). Biarylpropylsulphonamides are potent blockers of receptor desensitisation and are more selective for flip isoforms (Miu, Jarvie et al. 2001). Work by Gouaux and colleagues has proposed that cyclothiazide acts as an allosteric potentiator by binding at the interface between binding domains of adjacent subunits of the receptor, stabilising this interface and preventing desensitisation (Sun, Olson et al. 2002). Quirk and colleagues hypothesised that LY404187 and other biarylpropylsulphoamides may work in a similar fashion (Quirk and Nisenbaum 2003).

The differences in action, potency and subunit selectivity across different classes of compounds is an important feature of AMPA potentiators and one that may be exploited in clinical use. AMPA receptors are widely distributed across the brain and have a range of developmental and topographical expression patterns both in splice variants and subunit compositions (Hollmann and Heinemann 1994). It is reasonable to assume that differential modulation of different populations of AMPA receptors may have a range of behavioural and physiological consequences. Given the wide spectrum of conditions potentially treatable by AMPA modulation this seems to be a strong area of future drug development. Early evidence in imaging the effects on brain activity has already shown that different compounds can have distinct patterns of action across different brain regions (Fowler, Whalley et al. 2004; Jordan, McCulloch et al. 2005)

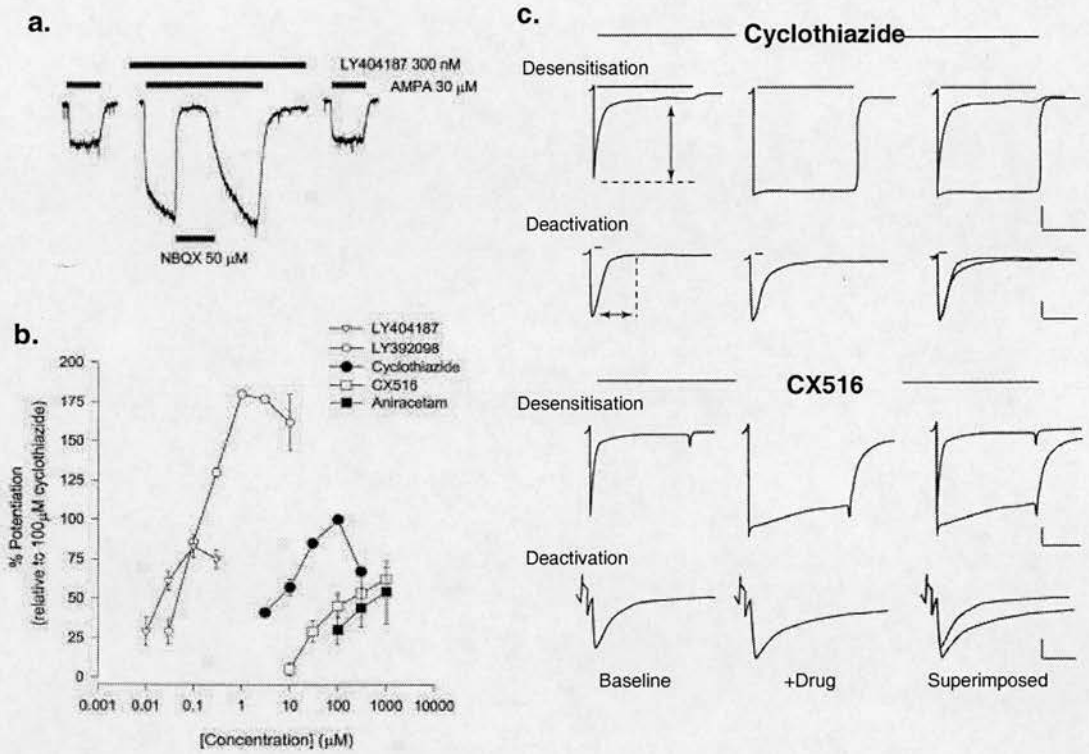


Figure 1.4 Pharmacological actions of AMPA potentiators

a) LY404187 is able to enhance the amplitude of AMPA receptor currents only in the presence of AMPA. This response in blocked by NBQX. b) LY404187 is able to potentiate glutamate evoked currents in cerebellar granule cells with over 100 fold higher more potency than older AMPA potentiators. c) The differential effects on two aspects of AMPA receptor kinetics of two classes of AMPA potentiator. Cyclothiazide blocks desensitisation but has little or no effect of deactivation. CX516 on the other hand has a smaller effect on desensitisation but has a large effect on the rate of deactivation of AMPA receptors. This phenomenon is seen across many AMPA potentiators each having specific pharmacokinetic profiles. From Gates et al. (2001) and Lynch (2004).

1.2.4 The AMPA potentiators LY404187 and LY450108

In this thesis I have studied the effects of two biarylpropylsulphonamide AMPA potentiators, LY404187 and LY450108. The former is one of the best characterised of Lilly's first generation of potentiators. LY404187 is ~100 times more potent than cyclothiazide at potentiating AMPA receptors in isolated cerebellar Purkinje neurons (Figure 1.4), potentiates hippocampal AMPA receptor mediated currents at nanomolar concentrations and is highly selective for GluR1-4 receptors with EC_{50} values ranging from 0.22-5μM (Gates, Ogden et al. 2001; Miu, Jarvie et al. 2001). LY404187 shows good brain penetration and can enhance synaptic transmission in rat prefrontal cortical neurons (Vandergriff, Huff et al. 2001), increase glucose utilisation and c-fos activation in rats (Fowler, Whalley et al. 2004). LY404187 has also been shown to promote the expression of the neurotrophin BDNF *in vivo* (Mackowiak, O'Neill et al. 2002) and has recently shown neurotrophic like properties *in vivo* (O'Neill, Murray et al. 2004) (Figure 1.6). Behavioural studies have shown that LY404187 is effective as a cognitive enhancer in a water maze test and can reduce immobility in the forced swim test (Quirk and Nisenbaum 2002). *In vivo* studies have shown that LY404187 is effective at enhancing BDNF expression, potentiating central synapses and promoting structural plasticity at doses of between 0.1mg/kg and 10mg/kg (Quirk and Nisenbaum 2002).

LY450108 has been less well described in the literature. It was initially developed as a clinical candidate and human pharmacokinetic studies have recently been made available (Jhee, Chappell et al. 2006). Although extensive pharmacological comparisons between LY450108 and LY404187 are not available, they have both been

shown to enhance the expression of BDNF *in vitro* and have been shown to have comparable neurotrophic effects in 6-OHDA lesion models of Parkinson's Disease (Lakics 2006; Messenger 2006).

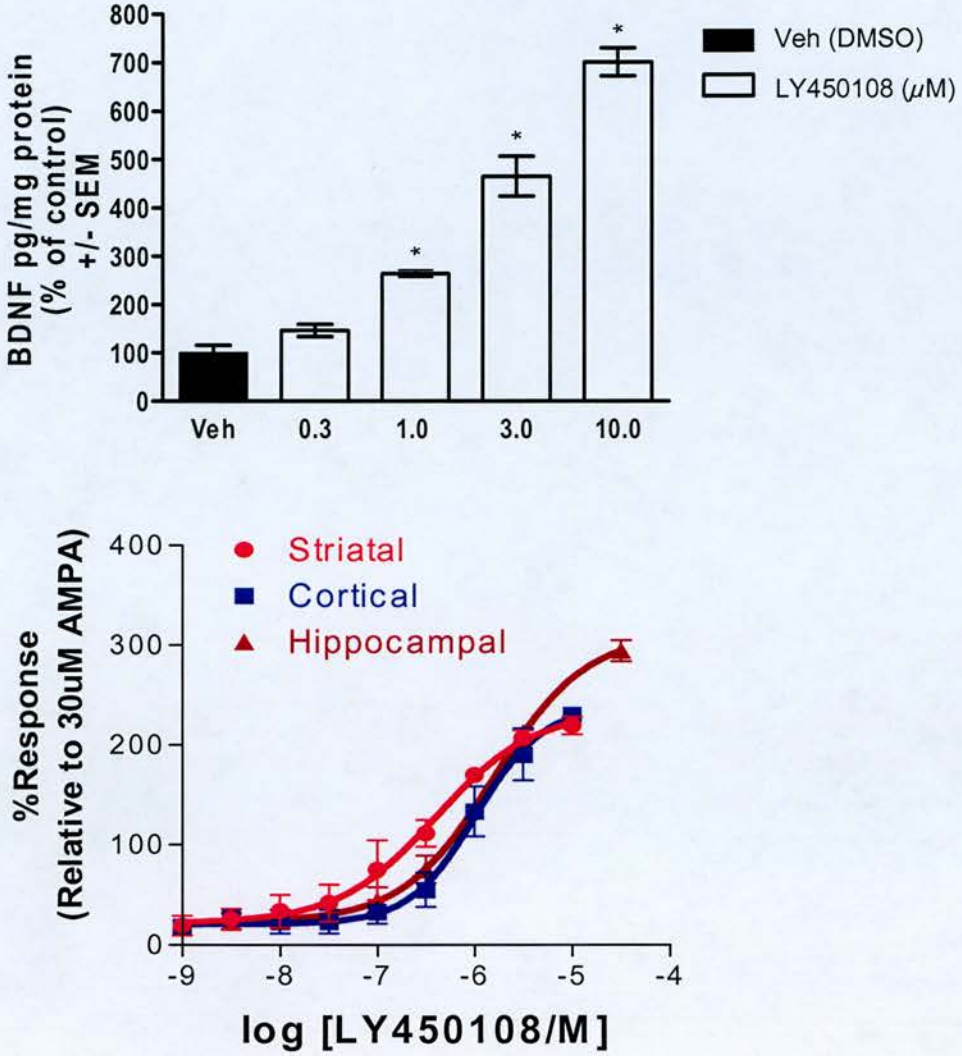


Figure 1.5 *In vitro* actions of LY450108

(Upper) LY450108 can induce the expression of BDNF in cortical neurons at concentrations between 1 and 10 μM. (Lower) The potentiation of native AMPA receptors in various cell types by LY450108 (μM range) is comparable to that of LY404187 (Quirk and Nisenbaum 2002). Figures taken from conference abstract (Neuroscience 2005 Washington D.C). Courtesy of M.J.O'Neill.

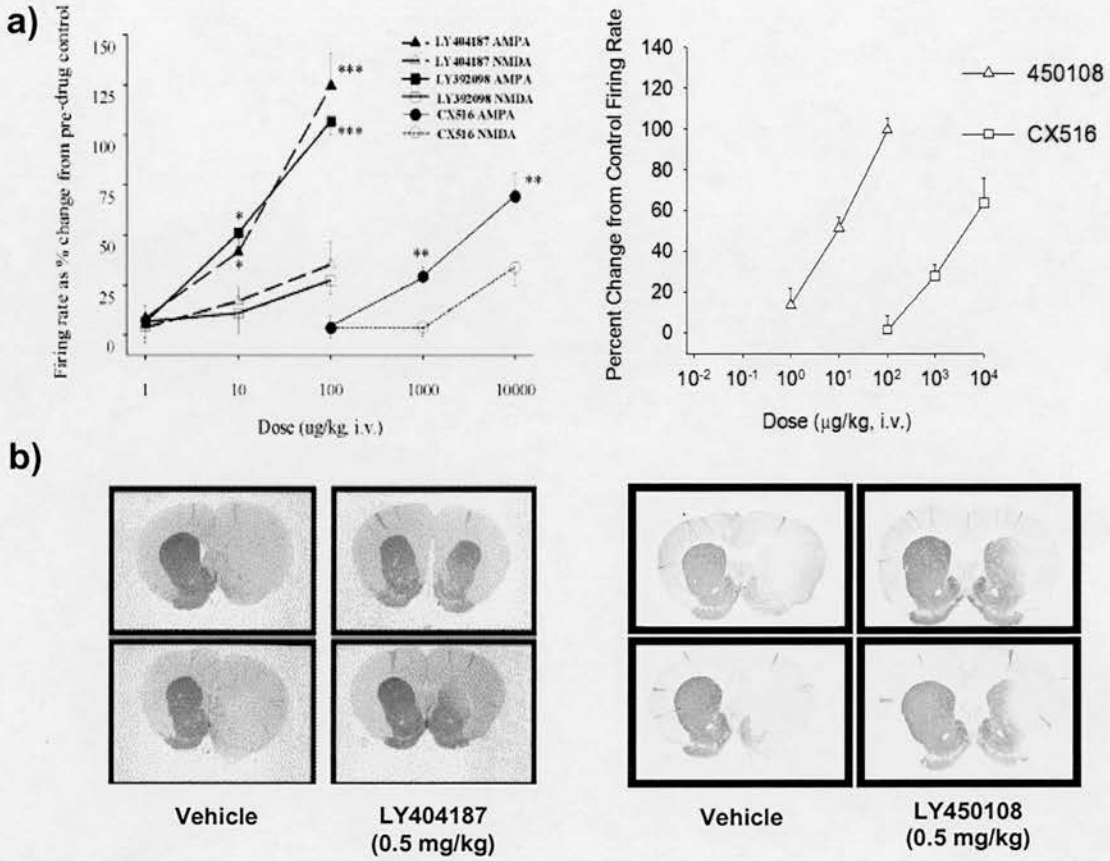


Figure 1.6 *In vivo* actions of LY404107 and LY450108

(a) Data showing the effect of AMPA potentiation on the firing rate of prefrontal cortical neurons in vivo. *Both LY404187 (left) and LY450108 (right) enhance the rate of firing in these cells with a 100-fold higher potency than CX516* (Quirk and Nisenbaum 2002) *(b) Neurotrophic effect of LY404187 and LY450108 in a rat 6-OHDA lesion model. Images are representative of a series of studies performed by O'Neill et al showing that AMPA potentiation can promote the recovery of TH immunoreactivity in the caudate nucleus following partial lesions of the nigro-striatal tract. The authors concluded that AMPA potentiation could enhance sprouting of surviving dopaminergic terminals within the caudate nucleus (O'Neill, Murray et al. 2004).*

1.2.5 AMPA potentiators as cognitive enhancers

AMPA potentiators were first conceived as cognitive enhancers and their ability to enhance memory and cognition remains by far their most well-documented feature. In strong support of the central hypothesis for AMPA potentiators AMPAkines have been shown to facilitate LTP induction (Staubli, Perez et al. 1994), promote memory formation (Staubli, Rogers et al. 1994) and olfactory learning (Larson, Lieu et al. 1995), reverse age-related memory impairments in rats (Granger, Deadwyler et al. 1996), improve working memory in non-human primates (Buccafusco, Weiser et al. 2004; Porrino, Daunais et al. 2005) and improve performance in recall tasks in humans (Ingvar, Ambros-Ingerson et al. 1997; Lynch, Granger et al. 1997).

AMPA potentiators have since been shown to have cognitive enhancing effects in many behavioural paradigms. The evidence shows that these compounds can enhance the formation of short term memory and show activity in certain behavioural models that are predictive of antidepressant properties (Li, Tizzano et al. 2001; Alt, Nisenbaum et al. 2006), there are also effects in human trials of schizophrenia (Marenco, Egan et al. 2002) although published research remains limited (Tuominen, Tiihonen et al. 2005).

AMPA potentiators also have an added feature that makes them interesting as potential therapeutic agents; they enhance expression of the neurotrophin BDNF. This property of AMPA potentiators has been demonstrated *in vitro* (Lauterborn, Lynch et al. 2000; Legutko, Li et al. 2001; Lauterborn, Truong et al. 2003) and *in vivo* (Mackowiak, O'Neill et al. 2002) and is thought to underlie the neurotrophic effects seen in both systems (O'Neill, Murray et al. 2004; Wu, Zhu et al. 2004; O'Neill, Murray et al. 2005).

Additionally, AMPA potentiation has been shown to enhance the rate of neurogenesis in the sub-granular layer of dentate gyrus (Bai, Bergeron et al. 2003). These observations have led to speculation that, as well as providing cognitive enhancement, AMPA potentiators may promote a long term neurotrophin-mediated therapeutic benefit in a number of neurological conditions (O'Neill, Bleakman et al. 2004; Alt, Nisenbaum et al. 2006).

1.2.6 AMPA potentiators and neurotrophins

The seminal observation that mouse sarcomas implanted into developing chick embryos caused nerve fibres from adjacent dorsal root ganglia to grow into the tumour, led to the isolation of a soluble low-molecular weight protein later identified as NGF (reviewed in Levi-Montalcini 1987). The subsequent work of Viktor Hamburger, Rita Levi-Montalcini and Stanley Cohen throughout the 50s laid the foundation for the study of the so-called neurotrophic factors. These are proteins capable of promoting cell survival, growth and synaptic plasticity throughout the developing and adult CNS and PNS. Many families are included in this group, including TGF-β, FGF, IGF, EGF, BMP and PDGF (Lessmann, Gottmann et al. 2003). The most important member of this group however is the neurotrophin family, which includes, in mammals, NGF, BDNF, NT-3 and NT-4/5. Each of these proteins signals through its cognate receptor, one of a family of cell-surface receptors with intrinsic tyrosine kinase activity, the so-called Trk (Tropomyosin Receptor Kinase) receptors: NGF has high affinity for Trk-A, BDNF has high affinity for Trk-B and NT-3 has high affinity for Trk-C, NT-4/5 has affinity for

Trk-B. There is also a degree of promiscuity, with NT4/5 also binding Trk-B. All neurotrophins also bind with a lower affinity to the p75 neurotrophin receptor (Rodriguez-Tebar and Barde 1988; Gentry, Barker et al. 2004).

Upon ligand binding, these receptors dimerise and auto-phosphorylate at specific intracellular tyrosine residues. This promotes the binding of small effector molecules that initiate signalling cascades resulting in a large number of physiological responses from cell survival, apoptosis, mitosis and neuritic outgrowth (Segal and Greenberg 1996; Bibel and Barde 2000; Segal 2003). Although neurotrophins are all essential for development of the CNS and PNS, as evidence with knock-out mice shows (Crowley, Spencer et al. 1994; Ernfors, Lee et al. 1994; Ernfors, Lee et al. 1994; Conover, Erickson et al. 1995), it is impossible to make many generalisations about the role of neurotrophins as any given protein will have multiple, often contradictory, actions across different regions or at different times during development (Lessmann, Gottmann et al. 2003). One example is the action of NGF and the low affinity neurotrophin receptor p75. NGF stimulates cell survival through Trk-A but can trigger cell death through p75. Other examples include the truncated forms of Trk receptors and the function of pro-form of neurotrophins (Segal 2003). As well as regulating developmental processes such as cell survival and axonal growth, the demonstration by Lohof and colleagues (1993) that neurotrophins (excluding NGF) could modulate synaptic transmission at the *Xenopous* neuromuscular junction opened up the study of the role of neurotrophins, particularly BDNF, in synaptic homeostasis and the modulation of transmission at mature synapses (Poo 2001).

Neurotrophins are capable of eliciting a range of physiological responses upon binding to their specific Trk receptor and are essential for the establishment and maintenance of a fully functional nervous system. In relation to the present study however, it is the role of neurotrophins in regulating axonal growth and promoting recovery and plasticity in the adult nervous system that will be explored in more detail.

The link between AMPA receptor activation and neurotrophin expression is well established (Zafra, Hengerer et al. 1990). The demonstration by Lauterborn that AMPA potentiation could induce the promotion of BNDF was in itself not surprising, but had very important repercussions in the field (Lauterborn, Lynch et al. 2000). This first demonstration of BNDF expression using AMPA potentiators has since been reproduced *in vivo* and *in vitro* using a number of other AMPA potentiators (Legutko, Li et al. 2001; Mackowiak, O'Neill et al. 2002; Lauterborn, Truong et al. 2003). One controversial aspect of this effect concerns the duration of the enhanced expression of BDNF. Lauterborn and colleagues showed that in organotypic hippocampal slice cultures BDNF expression returned to normal levels during 24 hours of continuous exposure to an AMPA potentiator. The explanation for this effect was that prolonged exposure to the AMPA potentiatior led to the degradation of the AMPA receptor complex (Lauterborn, Lynch et al. 2000). They subsequently showed that levels could be maintained at a higher level for a period of days if the compound was administered on alternate days (Lauterborn, Truong et al. 2003). However Mackowiack et al showed that *in vivo* BDNF mRNA levels were elevated even following two weeks of twice daily administration. These two observations can be reconciled when one takes into account differences in exposure to AMPA potentiators in the two systems. *In vivo* AMPA

potentiators tend to be metabolised very quickly and levels in the brain return within hours (O'Neill, Murray et al. 2005; Jhee, Chappell et al. 2006). Therefore, twice daily dosing could equate to the kind of on/off administration that can bypass degradation of AMPA receptors. At high doses some AMPA potentiators lose their potency displaying bell shaped response curves that could be explained by degradation of the AMPA receptor complex.

AMPA potentiator-induced expression of BDNF has been shown to be mediated both directly and indirectly by AMPA receptor recruitment of intracellular kinases. The non-receptor tyrosine kinase Lyn (Hayashi, Umemori et al. 1999) which associates with GluR2 subunits, was shown to activate MAP kinase pathways that lead to increased transcription of the neurotrophic factor *in vitro* (Wu, Zhu et al. 2004). Lyn's activation of MAP kinases was independent of Ca^{2+} influx through VGCC, indicating that Lyn may be activated by conformational change within the receptor subunit (given that GluR2 containing receptors are calcium impermeable) (Burnashev, Monyer et al. 1992; Hollmann and Heinemann 1994). Lyn's role in other aspects of AMPA receptor mediated neuroplasticity such as LTP and neurite growth has yet to be investigated but remains an interesting addition to the multiple pathways activated by AMPA receptor activity.

BDNF transcription is regulated by CREB, which can be phosphorylated by AMPA receptor activation (Perkinton, Sihra et al. 1999; Schurov, McNulty et al. 1999; Wu, Deisseroth et al. 2001). As well as BDNF, CREB also regulates the expression of a range of anti-apoptotic and neurotrophic genes such as Bcl-2 (Carlezon, Duman et al. 2005). Recent work indicates that other neurotrophins such as VEGF are also enhanced

by AMPA potentiation (Lakics 2006). What emerges is a profile of neurotrophic factors that can all be induced by AMPA potentiators.

One of the most well-characterised inducers of apoptosis is excitotoxicity (Olney and Ho 1970; Choi 1992) through glutamate receptors. Any mechanism that enhances glutamatergic transmission might be expected to exacerbate excitotoxicity. However Dicou and colleagues demonstrated that administration of CX516 was neuroprotective against ibotenate-induced cortical damage (Dicou, Rangon et al. 2003). A study on the neuroprotective effect of AMPA potentiation from the excitotoxic effects of high concentration of AMPA, corroborates this evidence (Wu, Zhu et al. 2004). The potentiation of AMPA receptors could specifically promote neurotrophic pathways rather than excitotoxic ones.

The neuroprotective effects of AMPA potentiation were also demonstrated in the 6-OHDA model of Parkinson's Disease (O'Neill, Murray et al. 2004; O'Neill, Murray et al. 2005). Histological and behavioural assessment of rats that had 6-OHDA-induced lesions of the nigro-striatal tract, and had been administered AMPA potentiators revealed significant improvement in drug-treated groups. The authors found evidence that the sprouting of nerve terminals in the striatum following the lesion was promoted following AMPA potentiator administration. Importantly they found that the compound was effective not only as a neuroprotectant (when administered during the degenerative phase) but could also induce histological changes when administered some time after the surgical insult, indicating neurotrophic mechanisms (Figure 1.6). This ability to induce structural change *in vivo* was a key finding and along with some evidence for the neurite growth-inducing properties of cyclothiazide (Fushiki, Matsumoto et al. 1995), were until

recently the only evidence supporting the neurotrophic effects of AMPA potentiators that has been suggested in a number of recent reviews (Limatola 2004, Alt et al 2005) .

1.3 AMPA potentiators and Structural plasticity

1.3.1 Neuroplasticity

Over the last 50 years our understanding of the brain's ability to adapt to its environment has changed greatly. We now know that the adult brain retains a large degree of plasticity, from synaptic plasticity, to the proliferation of stem cells, to large scale rearrangement of cortical networks and behavioural plasticity. Although most neuroscientists have a good working understanding of what neuroplasticity is in relation to their field, definitions vary: "the ability of the nervous system to change the effectiveness of [synaptic] transmission" or "structural, biochemical and molecular changes...supporting behavioural plasticity" (Cotman and Berchtold 2002)

The word plasticity was first used in relation to humans in 1890 by William James. He was the first to recognise that the most important feature of human behaviour is the ability to carry out meaningful change (James 1890, Cotman 2002). In 1969 Geoffrey Raismann first used the word to describe structural changes when describing sprouting in the septal nucleus or adult rats following lesions (Raisman 1969). Our understanding of the phenomenon has expanded to encompass a number of physiological and pathophysiological processes in the brain. There is a vast body of literature dealing with "neuroplasticity" (>17,000 hits on PubMed). One of the most

common definitions of neuroplasticity defines it as *the substrate for the brain's adaptive response to its environment.* Neuroplasticity is, therefore, the brain's primary function (as an extension of its role in maintaining homeostasis). A recent review divides plasticity into "fast", "medium" and "slow" processes (Peled 2005). This particular view is the most relevant to this thesis as it provides a useful framework in which to place the potential actions of AMPA potentiators.

This thesis is a study of how certain forms of plasticity can be promoted via glutamate synaptic transmission in order to halt or reverse certain pathological processes in some of the most common neurological conditions, via the induction of neurotrophin expression and long term structural and biochemical changes.

1.3.2 Principles of neurite growth

Elucidating the mechanisms by which neurons initiate, extend and retract their processes and ultimately how they select and establish meaningful synaptic connections with other neurons, is one of the most persistently relevant and challenging areas of neurobiology. Progress in this field has clinical as well as purely academic implications; the development of novel treatments for spinal chord injury (Schwab 2002), Parkinson's (Gash, Zhang et al. 1998; Lindvall, Kokaia et al. 2004) and Alzheimer's (Teter and Ashford 2002) and depression (Manji, Quiroz et al. 2003), all rely in some aspect on promoting structural neuroplasticity, whilst abhorrent neuronal sprouting is thought to underlie the pathology of some forms of epilepsy (Larner 1995).

The great neuroanatomist Cajal first proposed that the CNS was made up of neurons which extended fibres from the soma and made synaptic connections with neighbouring cells. This view, the 'neuronal doctrine', led to the field of study dedicated to the resolution of the mechanisms regulating this process (Levi-Montalcini and Hamburger 1951; Sperry 1963; Tessier-Lavigne and Goodman 1996; Jones 1999; Nikolic 2002). In the intervening century an extensive list of neurite growth-regulating factors has been compiled (Chilton 2006) and the task of describing this process is best approached "bottom-up", as so many distinct pathways converge at key regulatory stages. Neurite growth is a specialised form of cell motility where only one part of a polarised cell is involved. It is essentially a process of highly organised cytoskeletal reorganisation. Although cell types differ in the rate, regulatory cues and timing of neuritogenesis, the process has three main events; the initial budding, the formation of neuritic processes and finally the development of polarity with axons and dendrites (da Silva and Dotti 2002). The majority of evidence suggests that the principle driving force of this process is the actin cytoskeleton (Meyer and Feldman 2002). The principal site of this cytoskeletal reorganization is the growth cone, a highly specialised structure at the leading end of developing neurites first characterized by Cajal. The growth cone is a structure present at the leading edge of growing neurites that comprises large fan-like structures called lamelipodia and thin protruding processes called filopodia. The structure has adapted to sense the extracellular environment for molecules that direct the growth of the neurite (Kolodkin 1996; Korey and Van Vactor 2000). Growth cones are rich in filamentous actin (F-actin) which is constantly being polymerised at the leading edge of the growth cone in the filopodia, retrogradely transported and depolymerised in

the back of the cone (Suter and Forscher 1998). The rate of de-polymerisation can be regulated by an array of guidance cues, both secreted and cell-membrane bound (Tessier-Lavigne and Goodman 1996; Korey and Van Vactor 2000). One such mechanism suggests that actin polymerisation can be regulated by interactions between actin cytoskeleton and membrane bound receptors that grasp the extracellular matrix. The result is a shift towards polymerization and forward motion of the filopodia, a process called the "clutch" hypothesis (Mitchison and Kirschner 1988; Suter and Forscher 1998). Ultimately the regulation of neurite growth is much more complex, but the key regulators and intracellular cascades mediating this process are now beginning to be elucidated. All major signals that regulate growth cone dynamics converge at the level of actin polymerisation (Dickson 2002; Gillespie 2003). The main up-stream molecules that regulate this process are the Rho GTPase family of molecules (Nikolic 2002). Evidence for the role of these molecules in regulating the cytoskeleton first came from experiments in yeast (Adams, Johnson et al. 1990; Johnson and Pringle 1990) but it was the experiments of Ridley and colleagues using Swiss 3T3 fibroblasts that first showed that GTPases could directly regulate actin polymerisation (Ridley and Hall 1992; Ridley, Paterson et al. 1992). Rac1, RhoA and Cdc42 are the principal members of this family (Nikolic 2002). In neurons Cdc42 has been shown to induce neurite growth and RhoA to inhibit neurite growth whilst Rac1 has been shown to promote and inhibit neurite growth (Eaton, Auvinen et al. 1995; Luo 2000). Rho GTPases switch between active GTP-bound and inactive GDP-bound states under the regulation of guanidine exchange factors GEFs and GTPase activating proteins. The principal downstream targets have been identified as ROCK, Cdk5, N-WASP and Pak (Luo

2000). These kinases regulating actin assembly act principally through LIMK and its phosphorylation of ADF/cofilin. ADF/cofilin is a member of the family of actin-binding proteins that regulate the rate of actin de-polymerisation, the rate-limiting step in the treadmilling cycle of actin dynamics (Carlier, Laurent et al. 1997; Paavilainen, Bertling et al. 2004). Although multiple organism and cell-specific regulators of actin polymerisation exist, these principal molecules are evolutionarily conserved and are fundamental mediators of many processes involving cytoskeletal reorganization. Neurons are highly polarised cells often with extremely complex dendritic or axonal processes; throughout development and adulthood extracellular signals act on neurons to attract, repel, promote or inhibit neurite extension and branching. All these signals act by regulating the activation state of Rho GTPases which in turn regulate the rate of actin de-polymerization. These signals can be mediated by cell-cell interactions such as the semaphorins, diffusible molecules such as netrins or both, as is the case with ephrins. For the purpose of this thesis an in-depth assessment of these molecules is not relevant and many excellent reviews exist. The studies described in this thesis are concerned with the interactions between neurotrophins, synaptic activity and neurite growth.

1.3.3 Synaptic activity and structural plasticity

A potential mechanisms by which AMPA potentiators may treat neurological disorders is through the regulation of neurite growth by neurotrophins. Although early patterning of the CNS is mainly guided by molecular interactions between growing neurons, ECM and target-derived guidance cues (Tessier-Lavigne and Goodman 1996), there is a "critical" period of development extending into young adulthood were abnormal sensory experience as well as spontaneously generated electrical activity can radically affect the development of neuronal networks (Katz and Shatz 1996). The work of Hubel and Weisel in the late 1950 and 1960s (Wiesel and Hubel 1963; Hubel and Wiesel 1970; Hubel, Wiesel et al. 1977) on the visual system of cats and monkeys established the theory of a critical period during which sensory experience drives the formation of cortical circuitry. Thus, monocular deprivation induces a shift in the distribution of cortical cells responsive to the un-deprived eye of kittens (Hubel and Wiesel 1970), afferent activity is required to maintain dendritic stability in auditory nuclei (Deitch and Rubel 1984), NMDA blockade disrupts retino-tectal development in Xenopus tadpoles (Cline and Constantine-Paton 1989), glutamatergic signalling regulates dendritic development *in vivo* (Rajan and Cline 1998), visual activity promotes dendritic development *in vivo* (Li, Van Aelst et al. 2000) and sensory deprivation alters the dynamics of dendritic development in the rat barrel cortex *in vivo* (Lendvai, Stern et al. 2000).

Although a central role of synaptic activity has been established in the development and refinement of cortical circuitry, the actions of glutamate are varied and

often conflicting. Application of glutamate inhibites neuritogenesis in cultured embryonic rat motor neurons (Metzger, Wiese et al. 1998), whilst depolarization of PC12 cells enhances neurite growth in response to NGF (Howe 2003) and KA can induce neuritogenesis *in vitro* (Tsai, Chiu et al. 2002). In other examples, actin motility was inhibited in cultured rat hippocampal neurons by Ca^{2+} entry through VGCC and AMPA/Kainate receptors (Chang and De Camilli 2001).

Glutamate receptors can elicit a range of physiological responses so it is not surprising that the same holds true for the regulation of cytoskeletal dynamics. The majority of research in this area has been aimed at the role of afferent and spontaneous synaptic activity in regulating dendritic growth and development (Katz and Shatz 1996; Wong and Ghosh 2002). One of the principal mechanisms through which synaptic activity can shape dendrites is through elevation of intracellular calcium via VGCC, NMDA and AMPA receptors that regulate RhoGTPase activity through CaMKII (Wu and Cline 1998; Li, Van Aelst et al. 2000) or through CREB-mediated transcription via CaMKIV (Redmond, Kashani et al. 2002) and MAPKs (Wu, Deisseroth et al. 2001). There is also evidence for calcium independent activation of MAPK pathways via AMPA receptors via Lyn kinase (Hayashi, Umemori et al. 1999) G-proteins (Wang and Durkin 1995; Wang, Small et al. 1997). Although fast regulation of actin polymerization can be mediated directly through RhoGTPase activity via glutamate receptors, long-term structural change and homeostasis is mediated primarily through BDNF and Wnt signalling (Konur and Ghosh 2005). BDNF expression is linked to glutamatergic activity via calcium signalling and CREB phosphorylation (Zafra, Hengerer et al. 1990; Ghosh, Carnahan et al. 1994; Shieh, Hu et al. 1998; Tao, Finkbeiner et al. 1998) There

is very little evidence regarding the effect of AMPA receptor potentiators on neuritic growth. Fuskiki and colleagues showed moderate neurite growth in mouse CGN using the weak AMPA receptor potentiator aniracetam (Fushiki, Matsumoto et al. 1995). However the possibility that AMPA potentiation could induce neuritic plasticity has been suggested in a number of recent publications (O'Neill, Bleakman et al. 2004; O'Neill, Murray et al. 2004; O'Neill, Murray et al. 2005; Alt, Nisenbaum et al. 2006). Given the roles of synaptic activity and neurotrophins in the developing cytoskeleton it is not surprising that positive modulators of AMPA receptors could enhance neuritic development. The implications of these observations may be of critical therapeutic importance in a number of neurological disorders.

1.3.4 Structural plasticity in mood disorders

There is growing evidence to suggest that a number of mood disorders display some structural pathology that may contribute to the etiology of the disorders and may provide targets for pharmaceutical intervention (Manji, Quiroz et al. 2003). Studies have shown changes in hippocampal volume in a range of conditions including bipolar disorder, autism, PTSD and epilepsy. One particularly relevant case is that of major depressive disorder, which has been classically viewed and treated under the monoamine hypothesis of depression put forward over 40 years ago (Schildkraut 1965). Recent evidence has emerged implicating the neurotrophin BDNF and the generation of new neurons in the causes and possible treatment of depression (Duman, Malberg et al. 2000). AMPA potentiators are currently under investigation for the treatment of mood

disorders due to their ability to enhance BDNF expression, neurogenesis and their activity in classical animal models of depression (Bai, Li et al. 2001; Li, Tizzano et al. 2001; Bai, Bergeron et al. 2003; Alt, Nisenbaum et al. 2006).

1.3.5 Structural plasticity, BDNF and neurogenesis in depression

Depression affects approximately 120 million people worldwide. It is the leading cause of disability worldwide. It costs an estimated $83 billion per annum on the US economy (Gilbody, Sheldon et al. 2006) and by 2020 is expected to become the second largest contributor to the global burden of disease (WHO, 2006). The observation that imipramine (used in anti-histamine research) and iproniazid (an early anti-tubercular drug) could treat the symptoms of depression laid the foundations for 40 years of modern psychopharmacology. The discovery that both compounds increased central levels of noradrenaline led to the so called "Monoamine hypothesis of depression" (Schildkraut 1965; Coppen 1967). This theory, although it revolutionised the treatment of depression, does not explain a number of key observations. Chief amongst these is the phenomenon of the therapeutic lag associated with SSRI (selective serotonin uptake inhibitors) (Berton and Nestler 2006). Although central levels of monoamines are enhanced acutely following tricyclic or MAO inhibitor administration, alleviation of symptoms are not usually observed before a period of weeks (Sachar and Baron 1979). The development of sophisticated brain imaging techniques has revealed abnormalities in blood flow, glucose metabolism, reduced grey matter volume and enlarged ventricles

in a number of limbic and prefrontal cortical areas of depressed patients (Beyer and Krishnan 2002; Drevets, Bogers et al. 2002; Drevets, Price et al. 2002; Strakowski, Adler et al. 2002). Post-mortem studies on brain tissue have also found reduced cortical volumes and reduced neuronal and glial densities and volumes in a number of brain regions (Manji, Quiroz et al. 2003). It has been proposed that changes in brain physiology and structure may be due to prolonged exposure of the brain to circulating glucocorticoids. Glucocorticoids have been shown to regulate a number of plastic responses in the hippocampus, including the magnitude of LTP (McEwen 1999). Glucocorticoids can produce reversible atrophy of the dendrites of CA3 pyramidal neurons and suppressed neurogenesis (McEwen and Sapolsky 1995; Gould, McEwen et al. 1997). Dendritic atrophy is correlated with impairments in cognitive tasks and, importantly, both structural and behavioural impairments are reversed by treatment with the antidepressants tianeptine and phenytoin (Watanabe, Gould et al. 1992; Luine, Villegas et al. 1994; Conrad, Galea et al. 1996). In humans, stress has long been associated with the onset or exacerbation of depression (Anisman and Zacharko 1992; Post 1992) and approximately half of all depressed individuals have elevated Hypothalamus-Pituitary-Adrenal (HPA) activation (Holsboer, Gerken et al. 1986) which can be reversed following successful depression treatment (Rubin, Phillips et al. 1995). The link between stress and depression is far from clear however, as many people suffer from extreme or prolonged stress and do not develop depression.

Over the last decade Ronald Duman and colleagues at Yale have built a body of evidence supporting a role for CREB-induced BDNF expression and neurogenesis in the underlying pathology of depression as well as in the mechanisms of action of common

antidepressants (Duman, Heninger et al. 1997; D'Sa and Duman 2002). Electro convulsive therapy (ECT) is probably the most effective treatment for serious depression and has been used for over 60 years. Levels of Trk-B and BDNF have been shown to be elevated following ECT (Ernfors, Bengzon et al. 1991) and central infusion of BDNF has antidepressant effects (Siuciak, Lewis et al. 1997). Duman and colleagues showed that common antidepressants and electro convulsive seizures all increase BDNF mRNA (Nibuya, Morinobu et al. 1995). Furthermore they showed that antidepressants can enhance expression of CREB (Nibuya, Nestler et al. 1996), a transcription factor that regulates the expression of a number of anti-apoptotic and trophic factors including Bcl-2 and BDNF (Shieh, Hu et al. 1998; Tao, Finkbeiner et al. 1998; Finkbeiner 2000; Carlezon, Duman et al. 2005). Importantly they showed that the effects on BDNF were only apparent following chronic and not acute administration of antidepressants. Subsequent studies showed that the expression of CREB and BDNF had antidepressant properties (Chen, Shirayama et al. 2001; Shirayama, Chen et al. 2002). Further support comes from a post-mortem study in which subjects treated with antidepressants at the time of death had higher levels of CREB (Stewart, Chen et al. 2001) and BDNF (Chen, Dowlatshahi et al. 2001) in the hippocampus than non-treated patients.

Duman and colleagues have also shown that antidepressant and ECS can induce neurogenesis (Malberg, Eisch et al. 2000) as well as showing that expression of CREB can enhance cell proliferation (Nakagawa, Kim et al. 2002). Correlation however does not imply causation and in a follow-up study Malberg et al. showed that the antidepressant effects seen in an animal model of depression (inescapable shock) were abolished in animals that had received low-dose x-ray radiation, which reduces the rate

of neurogenesis (Malberg and Duman 2003). This was supported by another study which showed that neurogenesis was required for antidepressant–like effects (Santarelli, Saxe et al. 2003).

Although these studies provide much pre-clinical support for a cellular theory of depression based on the actions of BDNF and neurogenesis, a number of studies suggest that caution must be exercised. A recent study has suggested that in some areas of the brain BDNF may be necessary for the expression of stress-induced learned helplessness (Berton, McClung et al. 2006). Elsewhere, inducing learned helplessness in rats had no effect on cell proliferation in the hippocampus and reducing the rate of neurogenesis did not pre-dispose animals to developing learned helplessness (Vollmayr, Simonis et al. 2003). Clearly there remains much work to be done and an explanation of depression simply involving levels of BDNF is at odds with our current understanding of multi-system biology. However, the evidence suggesting that deficits in certain forms of plasticity contribute to the development of mood disorders is compelling and provides a framework to develop truly novel therapies.

AMPA potentiators, with their ability to penetrate the brain and increase expression of BDNF (Lauterborn, Lynch et al. 2000; Legutko, Li et al. 2001; Mackowiak, O'Neill et al. 2002; Lauterborn, Truong et al. 2003) and enhance rates of neurogenesis (Bai, Bergeron et al. 2003) are excellent candidates for the treatment of depression and other mood disorders (Skolnick, Legutko et al. 2001; Skolnick 2002; Alt, Nisenbaum et al. 2006).

1.3.6 AMPA potentiators in other CNS disorders

Schizophrenia is a promising target for glutamatergic-based intervention (Javitt 2004). Evidence for the involvement of glutamatergic transmission in the symptoms of schizophrenia includes the discovery that the potent psychotomimetic phencyclidine (PCP) was an NMDA antagonist. Trials with the PCP analogue ketamine have confirmed that single doses of NMDA antagonists can induce transient psychosis, cognitive impairment and motor disruption similar to those seen in schizophrenia (Glantz, Gilmore et al. 2006). Decreased NMDA receptor function may be one of the consequences of the development of schizophrenia but growing evidence from studying brain volumes, synaptic markers and neuropil markers suggests that synaptic disruption through unregulated apoptosis is involved (Glantz, Gilmore et al. 2006). Schizophrenia is in fact a condition that develops over time and can become progressively worse. There is evidence showing reduction in cell layer volumes in the anterior cingulate cortex and evidence of apoptosis and DNA fragmentation (Benes 2003). As well as potentially promoting structural plasticity that is deficient in some schizophrenia, AMPA potentiators may alleviate the synaptic deficits associated with structural degeneration and glutamatergic dysfunction (Lynch 2004). Two small scale trails using the AMPA potentiator CX516 revealed minors improvement in positive symptoms (Johnson, Luu et al. 1999; Marenco, Egan et al. 2002).

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disease that affects approximately 4.5 million people in the United States (Lledo, Alonso et al. 2006). Given that AD is characterised by progressive cognitive decline and cell loss in

cortical areas, AMPA potentiators may be well suited for alleviating some of the symptoms and possibly slow down the progression of the disease (Lynch 2004). There is evidence showing that the AMPA potentiator CX516 is able to enhance memory formation in aged adults (Ingvar, Ambros-Ingerson et al. 1997). Therefore enhancing glutamatergic transmission may help alleviate some of the cognitive impairment seen in AD patients (Lynch 2004). Cognitive function could also be enhanced by the facilitation of synaptic transmission by BDNF (Levine, Dreyfus et al. 1995). As with other conditions the possibility that enhanced levels of BDNF may help to promote cell survival and neuritic plasticity means that these therapies may help perhaps slow the progression of AD.

AMPA potentiators exhibit a range of physiological properties that are potentially of therapeutic value in a wide range of cognitive disorders. Through their acute ability to enhance synaptic transmission and facilitate the formation of memories and their potential neurotrophic actions on the structural integrity of dendrites and synapses, AMPA potentiators may be able to offer not only symptomatic relief but also potentially treat the underlying pathology of many common CNS disorders (figure 1.7).

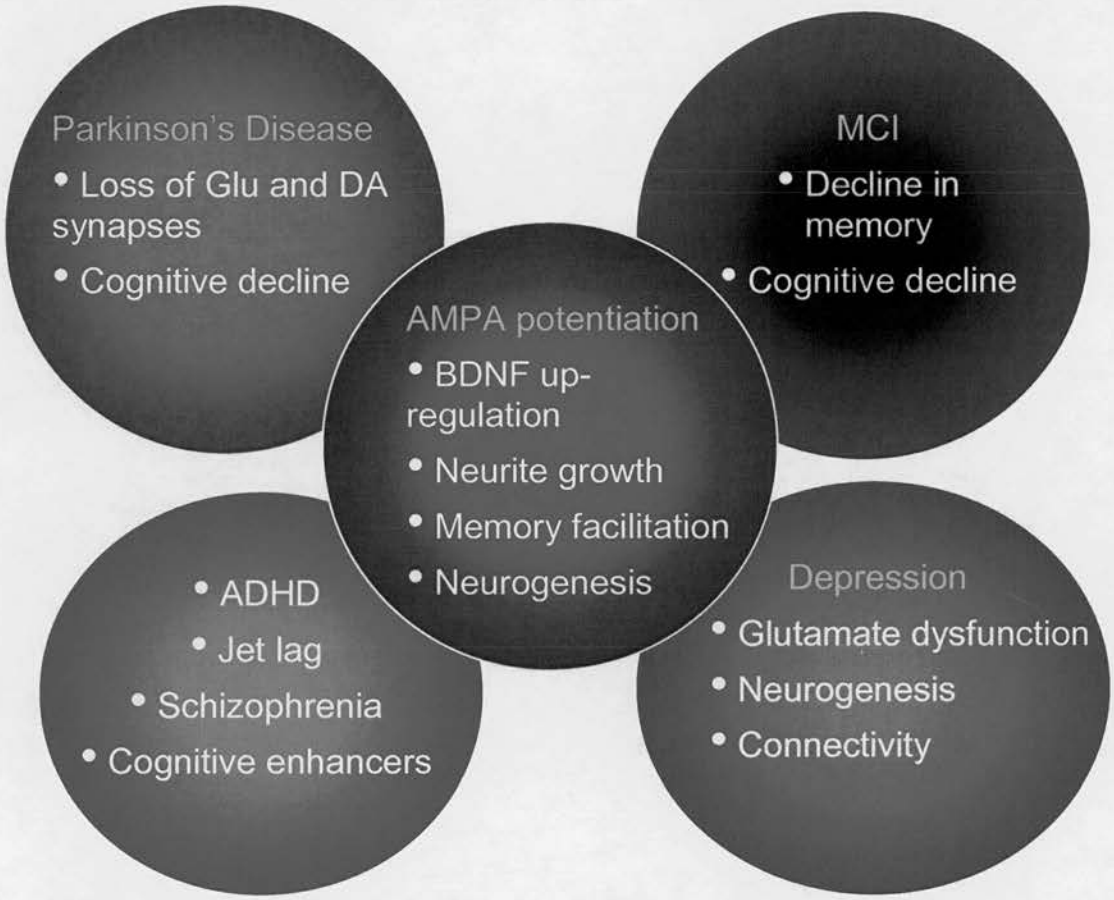


Figure 1.7 Clinical potential of AMPA potentiators

With their ability to promote synaptic transmission, induce certain forms of structural plasticity, increase the rate of neurogenesis and facilitate the formation of new memories, thus acting as cognitive enhancers, AMPA potentiators have the potential to be beneficial in a wide range of conditions (see text for references).

1.4 Aims of the Thesis

The aim of this thesis was to investigate the mechanisms of AMPA potentiators-induced neuroplasticity. I used a number of *in vitro* and *in vivo* techniques to examine multiple physiological, structural and biochemical measures of neuroplasticity. The specific aims of the three studies presented in this thesis are stated below.

1. Develop a statistically sound and reproducible, adequately powered *in vitro* model of neuroplasticity in SH-SY5Y human neuroblastoma cells. The primary endpoint is the change in the average length of neurites in response to AMPA potentiator administration. The secondary endpoint will be the changes in neurofilament expression measured by Western blotting. This model will be used to measure the effects and mechanisms of the AMPA potentiator LY404187 on neurite growth.

2. Measure the effect of subcutaneous administration of LY404187 on the formation of new synapses and the rate of neurogenesis in the hippocampal following unilateral partial lesions of the entorhinal cortex.

3. Monitor the persistence of biochemical and physiological changes induced by chronic administration of LY450108. Multiple endpoints will measure the biochemical signals associated with AMPA potentiation, the rate of neurogenesis

and neuronal development and the levels of the principal monoaminergic neurotransmitters in the neo-cortex, striatum and hippocampus.

2. Materials and Methods

2.1 Assessment of neurite growth

2.1.1 Introduction

Understanding the processes that regulate the growth and development of neurons is critical to our understanding of brain function and key to developing new clinical therapies. A powerful tool in this research is the study of neurite growth and development *in vitro*. From its early days in the study of neurotrophins (Levi-Montalcini 1987) to the sophisticated, real-time assays of today (Lohof, Quillan et al. 1992; Wang and Poo 2005) it has remained a popular and accepted method for elucidating the processes of neurite development. However, as yet there is no accepted standard measure of neurite outgrowth *in vitro* in particular when assessing population wide changes in average neurite length. The heterogeneity of cell populations and their processes renders automation of neurite measurements elusive. Existing techniques are either costly (Ramm, Alexandrov et al. 2003; Price, Oe et al. 2005) or use approximations of neurite length using stereological techniques (Ronn, Ralets et al. 2000). There is no consensus about which measure to use, with different groups using increases in average neurite length (Price, Yamaji et al. 2003), percentage of cells bearing neurites (Howe 2003), longest neurite per cell (Bamdad, Volle et al. 2004) and the percentage of cells bearing "long" neurites (Troller, Raghunath et al. 2004). In addition, some reports use pre-differentiated cells; a variety of treatments include retinoic acid (Adlerz, Beckman et al. 2003; Ruiz-Leon and Pascual 2003), tribromophenol (Rios, Repetto et al. 2003), aphidicolin (Price, Yamaji et al. 2003) and

staurosporine (Prince and Oreland 1997) with other groups reporting robust outgrowth in un-pre-treated cells (Shea, Cressman et al. 1995; Sanchez, Sayas et al. 2001; Tucholski and Johnson 2003; Troller, Raghunath et al. 2004).

For the purpose of our studies we selected an extensively used, human, neuroblastoma cell line that expressed AMPA receptors; SH-SY5Ys.

2.1.2 SH-SY5Y cells as a model of neurite growth

SH-SY5Ys are neuroblastoma cells of sympathoadrenal lineage that can be differentiated into cells with a neuronal phenotype in culture (Abemayor and Sidell 1989; Christnacher and Sommer 1995). SH-SY5Ys express AMPA receptor subunits (Figure 2.1) (Christnacher and Sommer 1995), BDNF and Trk-receptors (Olivieri, Otten et al. 2003; Ruiz-Leon and Pascual 2003) and have been used extensively as *in vitro* models of neuronal differentiation and neurite growth (Encinas, Iglesias et al. 2000; Ivankovic-Dikic, Gronroos et al. 2000; Simpson, Bacha et al. 2001; Price, Yamaji et al. 2003; Jamsa, Hasslund et al. 2004; Daniel, Mudge et al. 2005). In culture they display numerous cell phenotypes with around 30% percent of cells displaying a neuronal phenotype. Untreated SH-SY5Ys displaying a neuronal phenotypes are similar in appearance to SH-SY5Ys that have been treated with retinoic acid, although they are less numerous. Retinoic acid and NGF share common pathways in the induction of neuritic length (Corcoran and Maden 1999) and the co-administration of retinoic acid and BDNF induces extensive differentiation in SH-SY5Ys. AMPA activation leads to increases in BDNF (Zafra, Hengerer et al. 1990; Legutko, Li et al. 2001; Mackowiak,

O'Neill et al. 2002) and the choice was made not to pre-differentiate the SH-SY5Y cells so as not to interfere, *a priori,* with mechanisms that might be involved in the increase of neurite length following AMPA potentiation.

2.1.3 Cell culture

SH-SY5Y cells (ECACC No. 94030304) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated foetal calf serum (FCS) (Gibco), 2mM L-glutamine and 1% penicillin/streptomycin mix. Cells were cultured for up to 6 weeks (<passage 20 in accordance with ECACC guidelines). Optimum culture conditions were established using a range of seeding densities and incubation times. In neurite growth experiments, cells were plated in NUNC 6-well dishes at a density of 1 x 10^5 cells / well 24 hours prior to testing. Medium was replaced with fresh DMEM (2% FCS, 2mM L-glutamine) and appropriate compounds applied for 72hrs. All compounds were stored at -20°C in stock aliquots and fresh dilutions were made on each experimental day. After 72 hrs, cells were fixed using 4% PFA for 2 hrs before being processed for data collection.

2.1.4 Measuring neurite length

Neurite growth was assessed by taking a series of digital images of fixed cells and measuring the average length of all neuritic processes using the image analysis software Image J (NIH freeware) (Figure 2.1). The experimenter, blinded to treatment group, selected fields at random under a microscope and captured phase-contrast images at 200x magnification from each well (Leitz Laborlux microscope with Polaroid CCD camera running Polaroid DMC2 software). SH-SY5Ys have a heterogeneous population of cell phenotypes, which display a range of cell body shapes and process number and length. Because of high numbers of non-differentiated cells present in a population of SH-SY5Ys, images were only captured if they contained dispersed, neurite bearing cells. The system was checked for image compression and calibrated before use by taking horizontal and vertical images (200x) of a standard graticule. Neurites were traced manually and the length calculated. Neurites that extended past the edge of the field, crossed other neurites, touched other cells were not measured. All neurites were measured from the cell body to the extremity of the neurites. Branches, extending from neurites were not measured and all process shorter than 20μm (average width of cell body) were not included in further analysis.

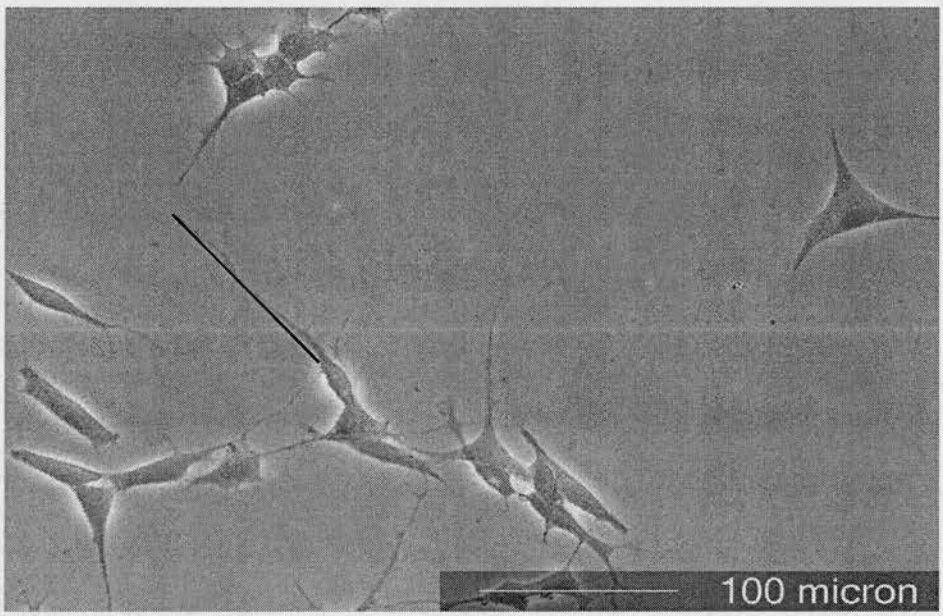


Figure 2.1 Manual tracing of neuritic processes in SH-SY5Ys

Processes were traced manually using Image J software. The length of the line is automatically calculated (in this case 102μm). All processes longer than 20μm were measured and used in statistical analysis.

2.1.5 Sampling, Power and Statistical analysis

Power analysis was used to ensure accurate sampling size in neurite growth experiments. The distribution of neurites yielded an average standard deviation of 20μm. The minimum detectable difference was set at 5μm (10% increase in length over control cells). The power and probability were set at the standard 0.8 and 0.05 levels. The test was designed to estimate the minimum sample size for ANOVA with 5 groups the size of a typical experiment. Calculations were carried out using Sigma Stat software package and yielded a predicted sample size of 379. The distribution of neurites in a population of SH-SY5Ys failed a normality test (K-S Dist. = 0.104, p = 0.002)) (Figure 2.2). Because of this, data was pooled into one population of 350-400 neurites collected over 4 experimental days and 8 independent wells and analysed using a Kruskal-Wallis analysis of variance on ranks followed by comparisons to vehicle treated cells using Dunn's post hoc analysis, as the group sizes were not equal across treatment groups. Significance was set at 95% confidence, $p<0.05$. For BDNF antibody and CNQX studies additional, multiple Mann-Whitney comparisons were run as a post-hoc test following analysis of variance. In this study p-values were corrected for multiple comparisons using the Bonferroni procedure. For purposes of clarity we have presented all neurite growth data as the mean average neurite length for the entire population in each group. Comparisons between the two methods for presenting the data are shown in Figure 3.1.6.

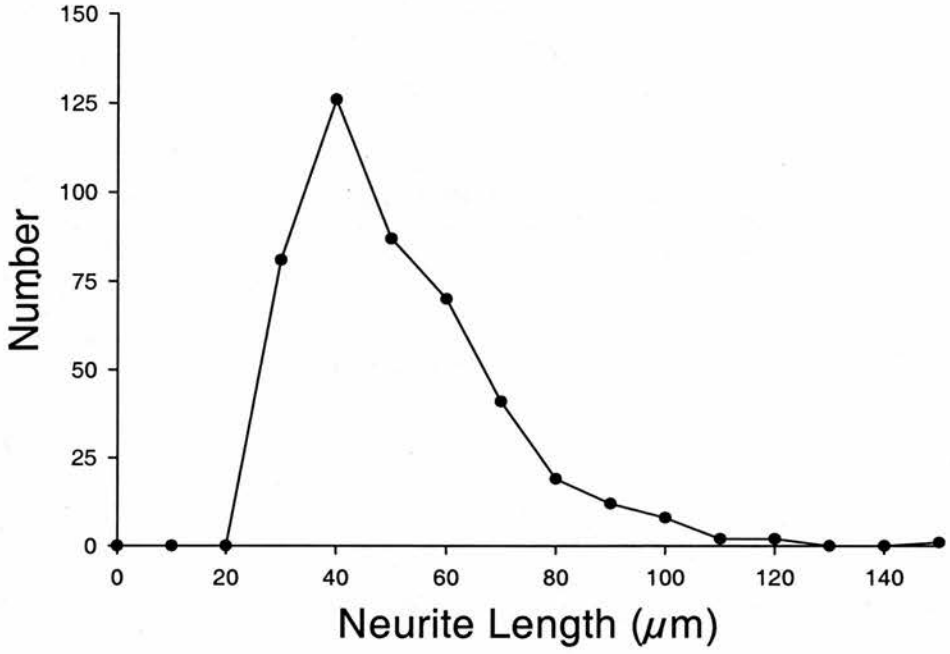


Figure 2.2 The distribution of neurite lengths in SH-SY5Ys

Frequency distribution analysis of neurites longer than 20μm collected from a population of SH-SY5Ys. The distribution of neurites failed a normality distribution test (see text) n>400 neurites.

2.2 Western Blotting

2.2.1 Introduction

Since its development, Western blotting has become one of the most commonly used techniques in biology (Burnette 1981). Western blots, named to differentiate from Southern blots (DNA) and Northern blots (RNA), rely on the separation of proteins of different mass on an acrylamide gel (SDS-PAGE) and the detection of the protein of interest using antibodies raised against protein specific epitopes. Although proteins can now be manipulated and imaged with a high level of temporal and spatial resolution and that Western blotting remains a semi quantitative measure. Nevertheless its technical simplicity, low cost and robustness ensure that it remains the best method for measuring large changes in levels of a protein of interest. The quality of the final blot depends in large part on the amount of protein present and the separation but primarily on the specificity and selectivity of the antibody.

2.2.2 Cell culture and protein extraction

In Western blot experiments cells were seeded at a density of 1 x 10^6 per 9cm dish and incubated in DMEM (2% FCS, 2mM L-glutamine) containing the appropriate compound. Following incubation, SH-SY5Y cells were washed with ice-cold PBS and lysed in 500μl NP40 lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH 7.4; 10 mM EDTA; 0.6% Nonidet P40; 1 mM Na_3VO_4; 10% glycerol; 10 μg/ml pepstatin; 1 mM

phenyl methyl sulfonyl fluoride). Cell lysates were incubated on ice for 30 mins before removing cell debris by centrifugation (15300g/10min/4°C) and protein concentrations determined using a modified Lowry method (Bio-Rad D2 Protein Assay kit; Bio-Rad Laboratories, Hemel Hempstead, UK).

2.2.3 Western Blotting protocol

10 μg of protein in standard loading buffer (25 mM Tris-HCl (pH 6.8); 0.8% SDS; 1% 2-mercaptoethanol; 4% glycerol; 0.01% bromophenol blue) was loaded on a 4-12% Tris glycine gel and separated by SDS-PAGE (200 mV for 45 min). Proteins were transferred to a PVDF membrane (Millipore (UK) Ltd, Watford, UK), and incubated in Tris Buffered Saline (TBS)-Tween (50 mM Tris-HCl; 150 mM NaCl; 0.05% v/v Tween-20) containing 5% albumin w/v for 1hr. Sequentially, membranes were probed with primary antibody; neurofilament (1:2000 anti-200kDa Neurofilament Heavy - Ab7795, Abcam), Trk (1:500, B-3 sc-7268, SantaCruz), β-actin (1:5000, Sigma, UK) and peroxidase-conjugated IgG (1:30000; Sigma, UK) before detecting with Enhanced Chemiluminescence Detection system (ECL plus; Amersham Biosciences UK Ltd., Little Chalfont, UK). Between each step membranes were washed three times for 5 mins with TBS-Tween. Chemiflourescence levels were corrected for background, normalised to actin loading controls and expressed as a fold increase above control cells (Figure 2.3).

2.2.4 Statistical analysis

The data obtained from the NF western blot was not amenable to standard statistical analysis due to the small sample size and the large variance (see results for further details). This finding influenced the design of subsequent western blot experiments. When studying the effect of LY450108 on the levels of pERK in the hippocampus larger group sizes were used. In this experiment (Figure 3.3.1) data was analysed by ANOVA followed by *post hoc* Student t-test with Bonferroni corrections. Data are presented as the mean ±SEM. Significance was set at $p<0.05$.

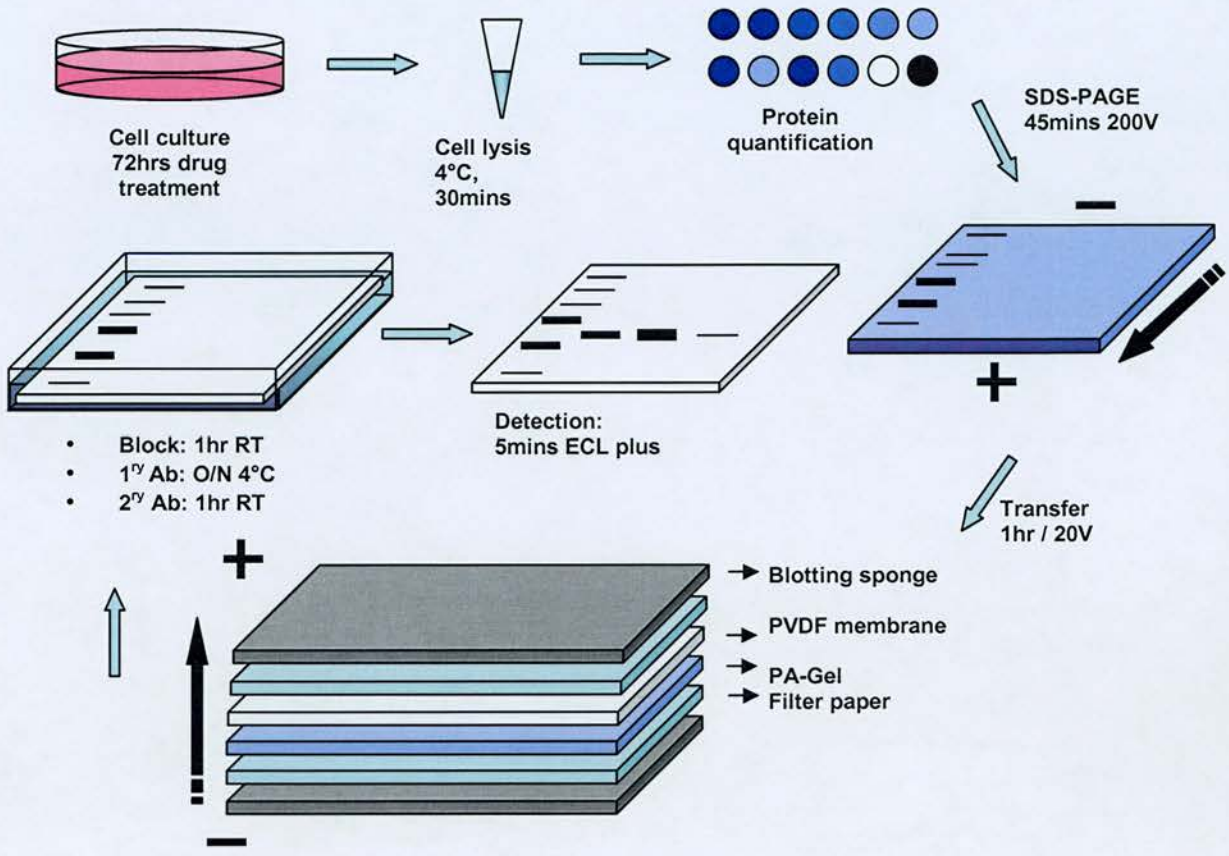


Figure 2.3 Schematic representation of Western Blotting methodology

Schematic representation of western blot methodology. Refer to text for full details of experimental protocol.

2.3 Investigations of the effects of AMPA potentiators *in vivo*

2.3.1 Housing conditions

Male adult C57BL/J mice (~20-25g) were housed in cages, randomised for drug treatment, in groups of six as long as possible throughout the study. Throughout the studies all procedures complied with Home Office regulations and condition and weight of the animals was controlled daily.

2.3.2 Drug treatment

Animals were administered twice daily injections of LY404187 or LY450108 via s.c injections. Each animal was weighed daily and given the appropriate volume to ensure 0.5mg/kg compound delivery at 10ml/kg injection volume. The vehicle for LY404187 was 10% ethanol, 3.75% hydroxypropyl-β-cyclodextrin and the vehicle for LY450108 was CMC. The drugs were made daily from frozen stocks. The study using LY404187 was performed at the University of Edinburgh and the dosing was carried out by the author. The study using LY450108 was carried out in the laboratories of Eli Lilly and dosing was performed by Dr. Tracey Murray.

2.3.3 Entorhinal Cortex Lesions

Surgical procedures described in Chapter 4 were all performed by Dr. Jill Fowler.

Lesions of the perforant path, either by excito-toxic cell death or mechanical sectioning of axons, results in synapse loss on dendrites of granule cells of the dentate gyrus. This is followed by an extended period of reactive synaptogenesis leading to an almost complete recovery of synapses occupancy on granule cells (van Groen, Miettinen et al. 2003). This model has been used extensively to investigate the processes of synapse degeneration and the plastic responses from gene expression to neurite sprouting and growth. The two main strengths of this model are that it allows the processes of degeneration and regeneration to be monitored in a topographically distinct area which is distant from the lesions site and that unilateral lesions allow for an internal control in the contralateral hemisphere.

Male adult C57BL/J mice (~20-25g) were housed in cages, randomised for drug treatment, in groups of six as long as possible throughout the study. Throughout the studies all procedures complied with Home Office regulations and condition and weight of the animals was controlled daily.

Animals were anesthetised in a perspex box using 3% halothane in a mixture of nitrous oxide and oxygen (70:30). Once anaesthetised, animals were transferred to a stereotaxic frame (David Kopf) and a face mask was placed over their snout and the halothane reduced to 1.5-2% for the remainder of the procedure. Rectal temperature was

monitored throughout the surgery keeping internal temperature around 37°C with the aid of heating lamp and a heated mat.

A midline incision was made in the scalp and the skin and muscle was retracted exposing the occipital bone down to the base of the skull. A 2μl Hamilton syringe was mounted to on the frame and the coordinates, determined from Bregma, were set at: AP 4.72mm and L 4.75mm and 17° from vertical. A hole through the skull, exposing the dura, was made using a dental drill. Forceps were used to peel back the dura exposing the dorsal surface of the cortex. The syringe was lowered onto the surface of brain and lowered a further 3mm. Ibotenic acid (α-amino-3hydroxy-5-isoazoleatic acid) 10mg/ml was then injected into the entorhinal cortex at a rate of 0.1μl/min until a total volume of 0.5μl was injected. 10 minutes elapsed following the injection to allow diffusion of the acid from the syringe tip. The syringe was removed and the skin was sutured, the animals were administered 200μl of saline and placed under observation until fully recovered.

2.3.3 Perfusion fixation

Animals were deeply anaesthetised with 5% halothane in a nitrous oxide and oxygen mix (70%:30%). Animals were then transferred to a mat fitted with a face mask over their snout and kept under heavy anaesthetic. When animals no longer responded to foot pinch an incision along the ventral midline and the skin and connective tissue was removed exposing the ribcage. The ribcage was raised and a butterfly needle was inserted into the left ventricle of the heart. Phosphate buffer (PB) containing 1% heparin

was then pumped at a rate of 3mls/min until the solution ran clear. Paraformaldehyde was then run through the animal until animal was fixed (~7mins). The head was removed and placed in paraformaldehyde for 24 hours. The skull was then removed and the brain placed in paraformaldehyde for a further 2 hours and finally placed in PB until processed for histology.

2.3.4 Paraffin embedding and tissue sectioning

3mm thick blocks of tissue were cut through the fixed brains using a cast metal mouse brain matrix. For the study investigating *in vivo* sprouting following ECL tissue blocks were taken through the entorhinal cortex and the hippocampus. The tissue used in this study was processed manually passing the tissue through progressive alcohols and into xylene. For the study investigating the effects of chronic administration of LY450108, tissue was taken from the hippocampus and processed automatically using a Vacuum Infiltration Processor, (VIP5, Bayer diagnostics, Newbury). Both sets of tissue were then manually embedded in paraffin blocks. 10μm sections were made through the entorhinal cortex and every tenth section used for histological assessment. 7μm thick sections were collected through the hippocampus. The sections were taken from the hippocampus at the level of the habenular nuclei.

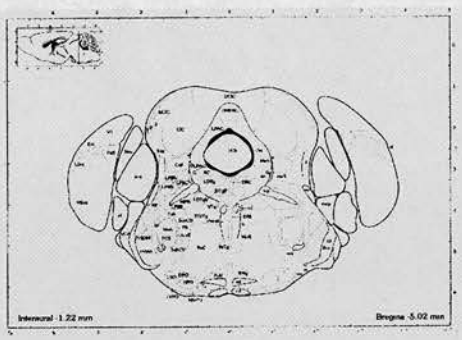
2.3.5. Quantification of entorhinal cortex lesion volume

For the ECL study sections through entorhinal cortex were stained with haematoxylin and eosin to visualise the damaged tissue and quantify the volume of lesioned brain. The tissue was processed as follows: 3x 2mins Xylene, 3x 2mins 100% ethanol, 1min 70% ethanol, 2min H_2O, 10mins Gill's Haematoxylin, 2x 5mins H_2O, 4mins Aqueous Eosin, 5mins Wash, 1min 70% ethanol, 3x 2mins 100% ethanol, 3x 2mins Xylene.

10 planes through the entorhinal cortex were taken from Paxinos and Watson's Mouse Brain Atlas (1986) and were used as a template on which to map out the lesioned area on each plane following examination using 5x light field microscopy (Figure 2.4). The distinction between lesioned and normal tissue was possible due to the well circumscribed lesions. These areas were then transferred to MCID software (3D-MCID) where the images were calibrated and 3D volumetric reconstructions were generated from which the approximate lesion volumes could be calculated (Figure 2.5). *A priori* exclusion criteria were set for animals which displayed lesions that spread into any area of the hippocampal formation and those which had no lesioned tissue or had lesions restricted to the needle tract. Such animals were excluded from all further analysis. Data were analysed with the appropriate statistical test and significance was set at $p<0.05$

Figure 2.4 Coronal Sections through entorhinal cortex

(Overleaf) 10 coronal plates through the entorhinal cortex taken from a mouse brain atlas (Paxinos and Watson 1986) used as a template on which to trace the extent of the lesion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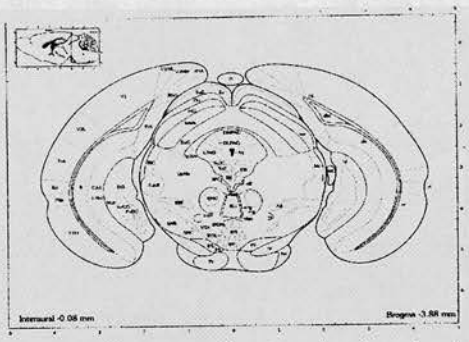
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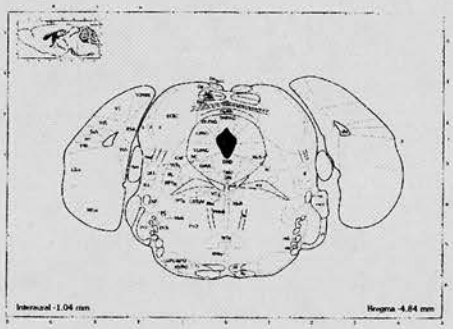
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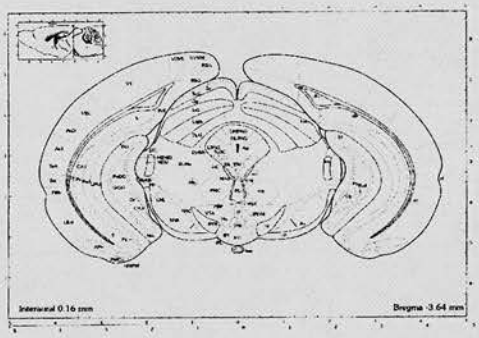
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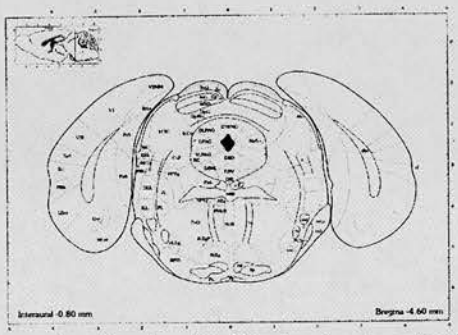
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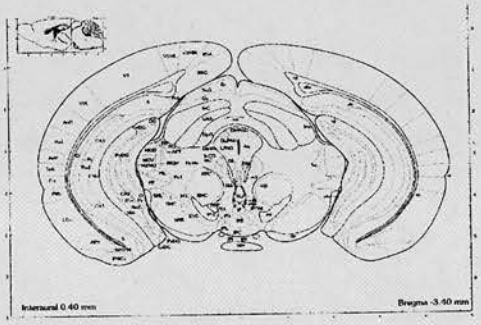
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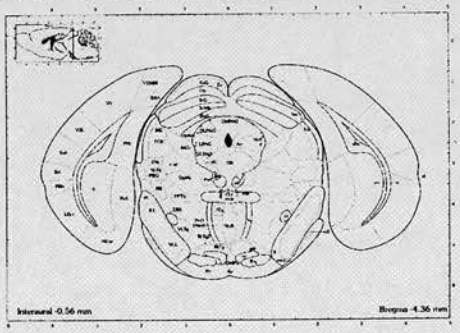
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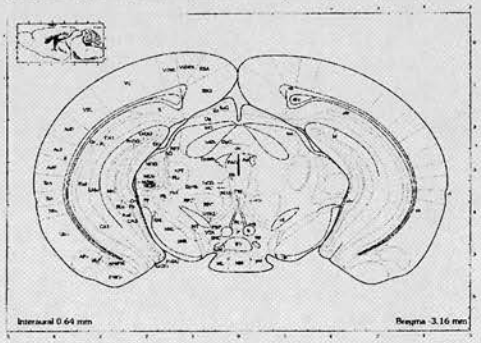
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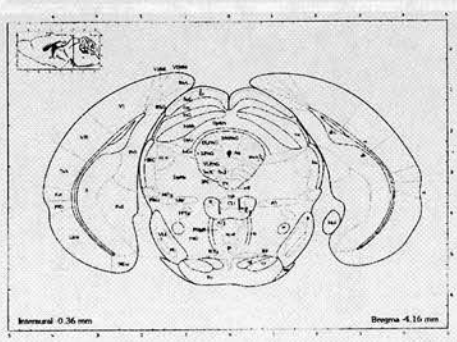
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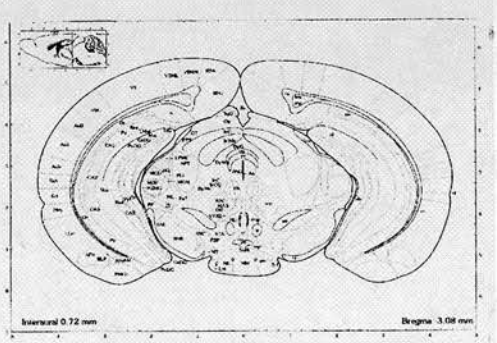
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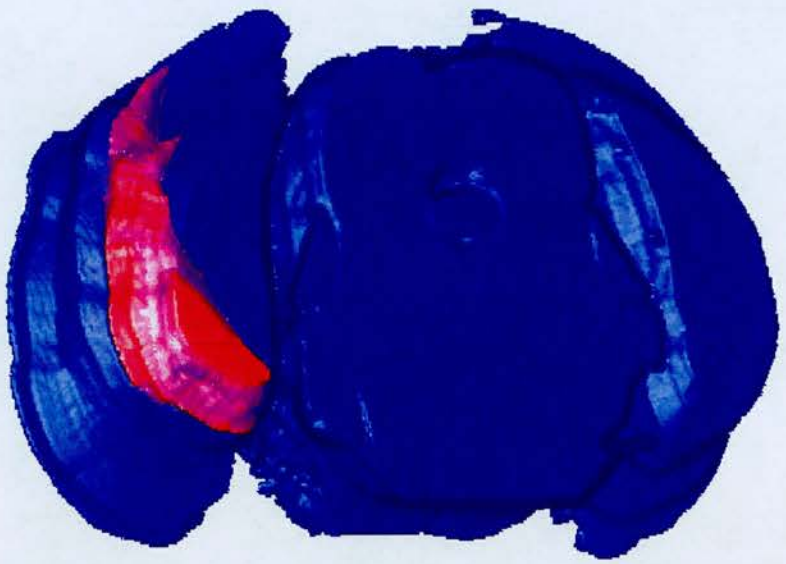


Figure 2.5 3-D reconstruction of entorhinal cortex lesions

3-D reconstruction of lesion (red) in the posterior 3mm of the entorhinal cortex (blue). The reconstruction allowed an estimate of the total volume of the lesion.

2.3.6 Immuno-staining of mounted sections

Mounted sections were de-waxed and processed for immuno-staining using the protocol described below (Figure 2.7). Antigen retrieval steps were incorporated for BrdU, NeuN, ERK 1/2 and CREB immuno-staining to improve the signal (30mins Citrate buffer 100°C. PT-Moudule, Lab Vision). For each antibody, blocking steps were carried out according to the manufacture's instructions. For NeuN an additional blocking step was performed using a specific "Mouse on Mouse" (M.O.M) blocking agent (Vector Laboratories). Optimum working dilutions and incubation times were determined using serial dilution curves (Table 2.6). All sections were incubated with primary antibody overnight at 4°C. The specificity of each antibody was assessed using primary antibody negative controls. Double labelling of BrdU and NeuN was performed by co-incubation of both antibodies at 4°C overnight. Appropriate secondary antibodies were used at manufacturer-recommended dilutions (Vector ABC kit, Vector laboratories, Peterborough). BrdU, pERK 1/2 pCREB and synaptophysin antibodies were developed using horseradish peroxidase conjugated secondary antibodies and DAB as the chromagen substrate. NeuN used Alkaline Phosphatase conjugated secondary antibody and Vector-Blue (Vector Laboratories) as the chromagen substrate (Table 2.6). This allowed a more diffuse staining pattern than that obtained using DAB, which was required during double labelling experiments.

Antibody	Species	Concentration	Conjugate	Chromagen
BrdU	Rat	1:100	HRP	DAB
NeuN	Mouse	1:100	AP	VectorBlue
pERK 1/2	Rabbit	1:100	HRP	DAB
pCREB	Rabbit	1:100	HRP	DAB
Synaptophysin	Rabbit	1:300	HRP	DAB

Table 2.6 Antibody dilutions for immuno-histochemical studies

Source, species, working dilutions and developing conditions for primary antibodies. All secondary antibodies were used at the manufacturer's recommended dilutions.

2.3.7 Quantification of immuno-staining

The staining pattern for BrdU, pERK 1/2 and pCREB was easily amenable to quantification by counting the number of positive cells per slide. The average number of positive cells over 2 sections per animal was used as the end point. pERK 1/2 $^{+}$ cells were readily counted in the dentate gyrus and CA1, CA2 and CA3 regions while the number of diffusely stained pCREB cells in the CA1, CA2 andCA3 regions was such that reliable quantification of these cells was not possible. $BrdU^{+}$ cells were clearly visible in the subgranular layer of the dentate gyrus and double labelled cells were clearly identifiable under high magnification due to the nuclear stain of BrdU and the predominantly cytosolic staining of NeuN

Synaptophysin immunoreactivity was assessed by measuring the optical density at 10 points along the middle molecular layer of 3 regions of the dentate gyrus; dorsal, medial and ventral. Readings for background OD were taken from the corpus callosum and subtracted from the DG readings (see section 3.2 for details).

2.3.8 Statistical analysis

Entorhinal cortex lesions and synaptophysin degeneration

The pilot experiment was aimed at determining the time course of synaptophysin degeneration in the dentate gyrus. To determine whether there was any significant difference in the ratio of ipsilateral and contralateral synaptophysin levels over time the data were analysed by ANOVA followed by post hoc Student's t-test with Bonferroni corrections for multiple comparisons.

In the LY404187 study we set out to determine whether administration of LY404187 (0.5mg/kg) had any significant effect on synaptophysin levels in the denate gyrus at 14 and 28 days post lesion. To determine whether there was any significant difference in lesion volume between LY404187 (0.5mg/kg) or vehicle treated groups at 14 or 28 post lesion, independent, two-tailed unpaired Student's t-test were performed on both data sets. To determine whether there was any significant difference in synaptophysin immuno-reactivity between LY404187 (0.5mg/kg) or vehicle treated groups, independent, two-tailed unpaired Student's t-test were performed on both data sets. To determine if there was any significant correlation between the size of the lesion and the levels of synaptophysin independent linear regression analysis was performed on each dataset. The final end point of the study was the level of neurogenesis in the dentate gyrus. To determine whether there was any significant difference in the number of BrdU positive cells in the sub-granular layer between LY404187 (0.5mg/kg) or vehicle treated groups, independent, two-tailed unpaired Student's t-test were performed on both data sets. All data are presented as either raw data of mean ±SEM. Significance was set at $p<0.05$.

Biochemical changes associated with LY450108

We set out to determine whether chronic administration of LY450108 can induce significant changes in number of pERK and pCREB +ve cells. We carried out ANOVA followed by *post hoc* Student t-test with Bonferroni corrections for multiple comparisons on both data sets. Data are presented as raw data or mean ±SEM. Significance was set at $p<0.05$.

We also set out to measure whether chronic administration of LY450108 can alter the rate of neurogenesis and neuronal development in the sub-granular layer of the dentate gyrus. To determine whether 3 days, 10 days or 17 days LY450108 administration (0.5mg/kg) can induce changes in the number of BrdU +ve cells multiple independent two-tailed unpaired Student t-tests were carried out on each data set. To determine whether 3 days, 10 days or 17 days LY450108 administration (0.5mg/kg) can induce changes in the ratio of NeuN+/BrdU+ cells to NeuN–/BrdU+ cells, multiple independent two-tailed unpaired Student t-tests were carried out on each data set. All data are presented as raw data. Significance was set at $p<0.05$.

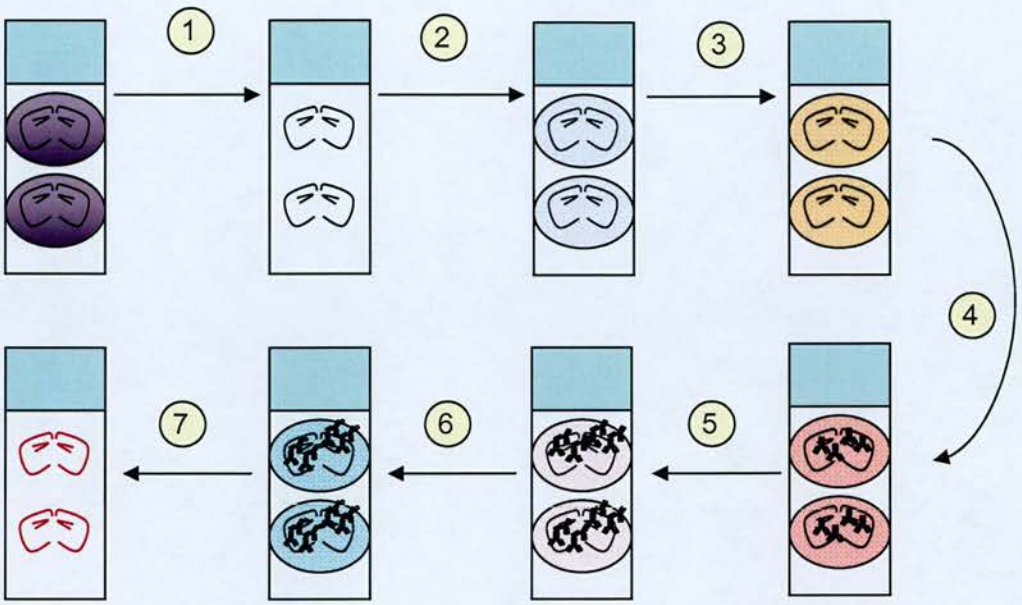


Figure 2.7 Schematic representation of Immuno-histochemistry methodology

Standard protocol for immuno-staining of paraffin embedded sections: 7µm sections were taken through the hippocampus at the level of the lateral habenulae and processed as follows. See text for details of each step.

Step 1: Sections were incubated in citrate buffer for 30mins at 100°C to remove paraffin wax and antigen retrieval (see-text) Step 2: Endogenous peroxidases were quenched using 0.3% H_2O_2 for 30mins. Step 3: Sections were blocked with serum from appropriate animal for 1 hour. Step 4: Sections were incubated in primary antibody over night at 4°C. Step 5: Sections were incubated in secondary antibody for 30mins at RT. Step 6: Sections were incubated with ABC horseradish-peroxidase or alkaline phosphatase complex. Step 7: Sections were developed using DAB for ~6mins or Vector Blue for 15mins Sections were washed 3x 5 mins with PBS between all steps (except 3 and 4).

2.6 Monoamine analysis by High Pressure Liquid Chromatography

2.6.1 Introduction

The use of chromatography as the principal technique for the separation, identification and quantification of components of mixtures became widespread in 1930s and 40s despite having been first used by the Russian botanist Micheal Tswett at the beginning of the 20th century. The basic principal of liquid chromatography is the process of separating and purifying substances dissolved in a mixed solution by slow passage through or over a surface of adsorbing material, making use of differences in absorbtion of the constituents to separate the components either as (coloured) bands or spots or by differences in speed of travel when washed through the adsorbing material. High Pressure Liquid Chromatography (or High Performance Liquid Chromatography) passes a solvent containing the mixture (mobile phase) under high pressure through a column of tightly packed beads (solid phase) to separate components either by size exclusion, affinity separation, ion-exchange or polarity. HPLC represented a significant improvement on previous liquid chromatography techniques allowing the rapid and sensitive separation of a mixture into its components. The separated components are then passed through a detector (UV, Refractive index or fluorescence) and identified in real time. The resolution of the system is dependent upon the extent of interaction between the solute components and the stationary phase which can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC

acquires a high degree of versatility and can easily separate a wide variety of chemical mixtures.

2.6.2 Reverse-phase HPLC with electrochemical detection

Reverse phase HPLC is one of the most common forms of the technique wherein the mixture of interest is dissolved in a polar solvent, in this case acetonitrile, and passed through a column of hydrophobic beads. The polarity of the individual components in the mixture will determine the retention time in the column and thus allow for separation along the whole column. The components then pass through the detector electrode which measures the current resulting from oxidation/reduction reaction of the analyte. Since the level of the current is directly proportional to the analyte concentration, this detector could be used for quantification. Although this form of detection is not very versatile its sensitivity to levels of phenols, catecholamines, nitrosamines, and organic acids is in the picomole (nanogram) range. By calibrating the detection software to concentration ranges of a number of catecholamines involved in the metabolism and catabolism of the principle monoaminergic neurotransmitters the experiment was designed to detect changes in the levels of these important compounds in key areas of the brain following long term exposure to an AMPA potentiator.

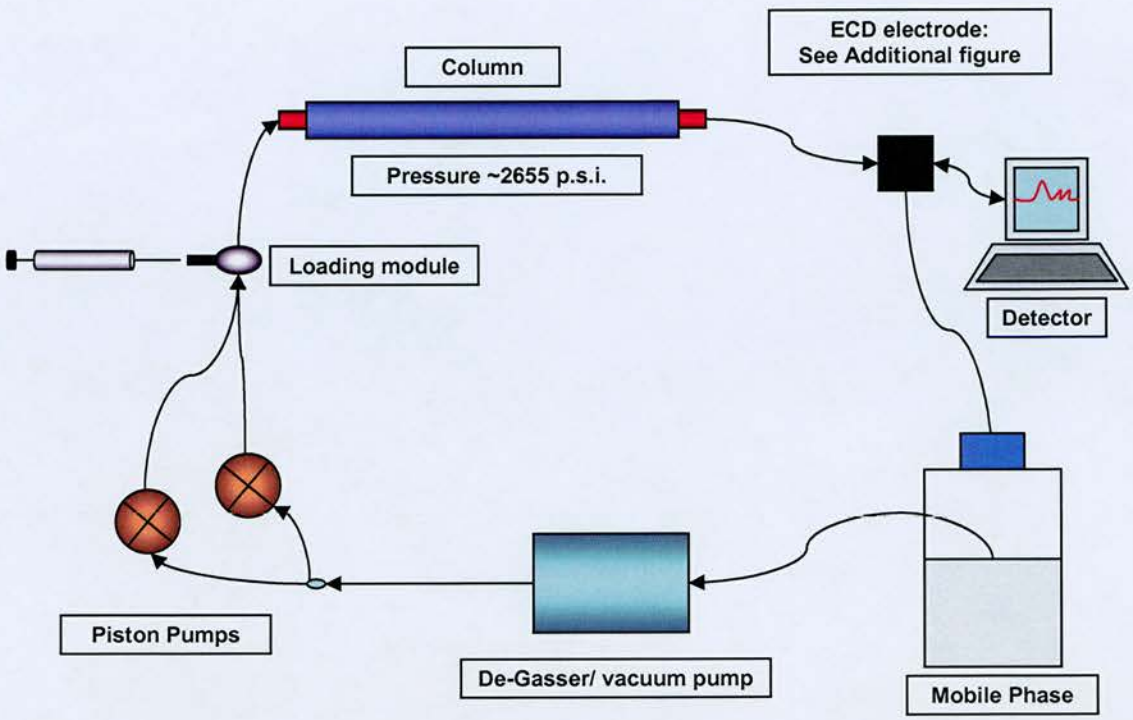


Figure 2.8 Schematic representation of HPLC methodology

The sample is loaded into the loop and run through for 20mins. The detector then gives readout of the concentration for each compound. The process of electrochemical detection (measuring redux currents) means that the system can be a closed loop where each compound can only be detected once.

2.6.3 Experimental protocol

Standard curves for each compound of interest were established by injecting 50μl PCA (60%) containing a mixture of the compounds at 2ng/μl, 0.5ng/μl and 0.125ng/μl through the column. The mixture contained Noradrenaline, DOPAC, Dopamine, 5HIAA, HVA, 5HT and the internal control NW-5HT. Each compound was identified as a separated peak with a distinct retention time.

Tissue from the neocortex, striatum and hippocampus was dissected from fresh mouse brain and snap-frozen in isopentane on dry ice and stored at -70°C until use. The wet weight of each sample was measured before being sonicated for 10s in ~100μl 60% perchloric acid (PCA) containing 0.1ng/μl NW-5HT. The sample was then spun down and the supernatant was run through the column (50μl). Concentrations were then obtained for each compound of interest and the final concentration of compound per mg of tissue was calculated.

2.6.4 Statistical analysis

In these experiments we aimed to measure whether chronic administration of LY450108 (0.5mg/kg) produced significant changes in the levels of aminergic neurotransmitters and their principal metabolites in three areas of the brain. The data was analysed by performing three, independent, two tailed unpaired Student t-tests for each time point. This test was performed for each metabolite in the cortex, hippocampus and striatum. All data are presented as mean ±SEM. Significance was set at $p<0.05$.

3. Results

3.1 LY404187 and neurite growth

3.1.1 Aims

The aims of this study were to establish a model for the measurement of neurite growth in the human neuroblastoma cell line SH-SY5Y. This model was then used to investigate the effects on neurite growth of the AMPA potentiator LY404187.

3.1.2 Establishment of neurite growth assay

The methods employed in the measurement of neurite growth are numerous and varied. To establish a reproducible and statistically sound assay we carried out pilot studies to determine the ideal working protocol for a number of experimental parameters

Differentiation of SH-SY5Ys

SH-SY5Y cells are a highly heterogeneous cell type. Figure 3.1.1 shows a typical field captured from cultured SH-SY5Y cells after 72 hours in culture. Using a variety of differentiation factors it is possible to increase the number of neuronal type cells and enhance considerably the average length of neurites. However in any given population of SH-SY5Y cells under standard culture conditions, there is a clear sub-population of cells that display neuronal phenotypes and a number of studies have been published that use pre-differentiated SH-SY5Ys. Experiments were carried out using retinoic acid (RA) to induce differentiation in these cells (data not shown). However I

decided against using RA for two reasons. First, although the number of neuronal cells was increased in RA treated cells, the phenotype of these cells was indistinguishable from the neuronal cells present in SHSY5Y cells under standard conditions (Figure 3.1.2). Second, there is evidence suggesting that RA and NGF exert their effects though the same mechanism of action. I had hypothesised that LY404187 may exert its effects of neurite growth through BDNF, therefore the decision was taken not to involve multiple growth factor pathways when they were under investigation.

Sampling

In view of the heterogeneity of SHSY5Y cells, it was important to determine that an accurate sample was being taken. By measuring the average neurite length from a population of cells collected from successive, randomly selected fields, I determined the number of fields that were required to ensure that any additional fields did not alter the average of the whole sample. Figure 3.1.3 summarises the data from three independent experiments each representing approximately 100 neurites in total. I determined from this study that between six and eight fields were necessary to ensure accurate population sampling. To balance the need to ensure accurate population sampling and the logistical issues of time and information storage whilst also ensuring adequate population sizes, in all subsequent experiments 8 fields per well were taken.

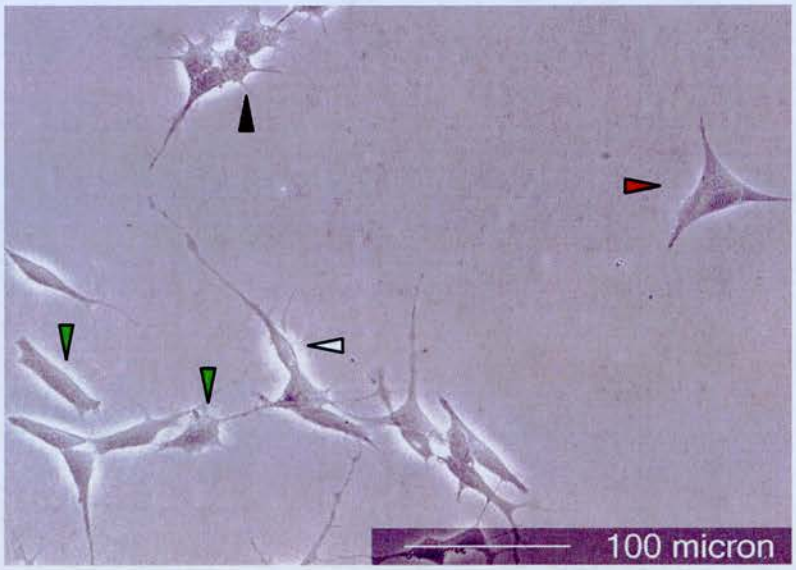
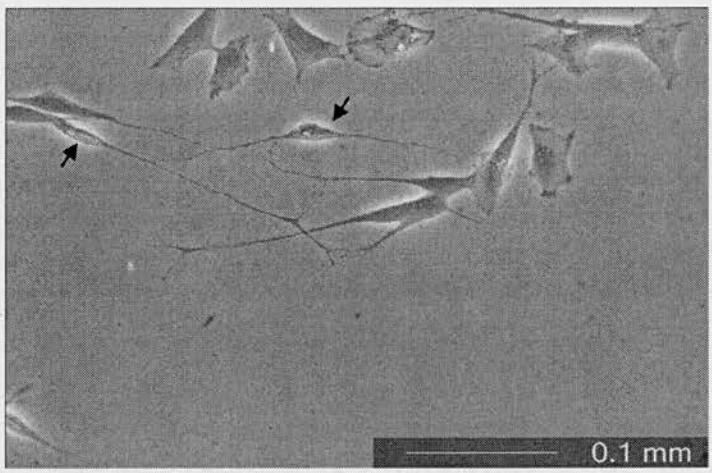


Figure 3.1.1 Heterogeneous populations of SH-SY5Ys

SH-SY5Ys display multiple phenotypes after 72hrs in culture. Round with multiple short processes (black arrow), Cells with fewer processes but no polarity (red arrow), neuronal-type cell with one or two long, thin processes and flattened cell body (white arrow) and miscellaneous cells exhibiting a range of non-neuronal phenotypes (green arrows).

a)



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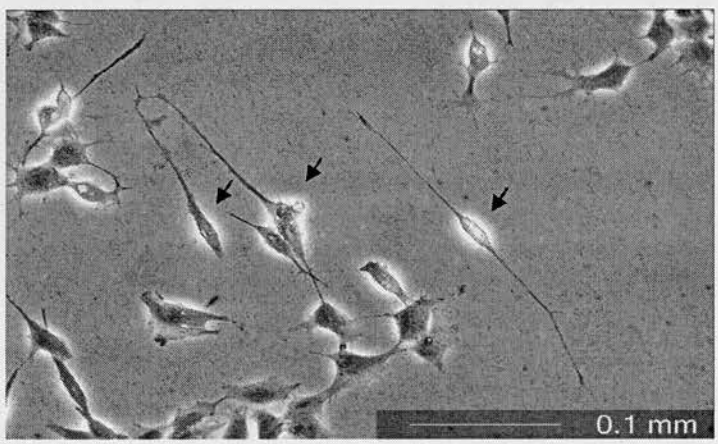


Figure 3.1.2 Differentiation of SH-SY5Ys

SH-SY5Ys treated with a) RA 100nM for 72 hrs and b) untreated for 72hrs. In both cases differentiated, neuronal-like cells are present, displaying long neuritic processes.

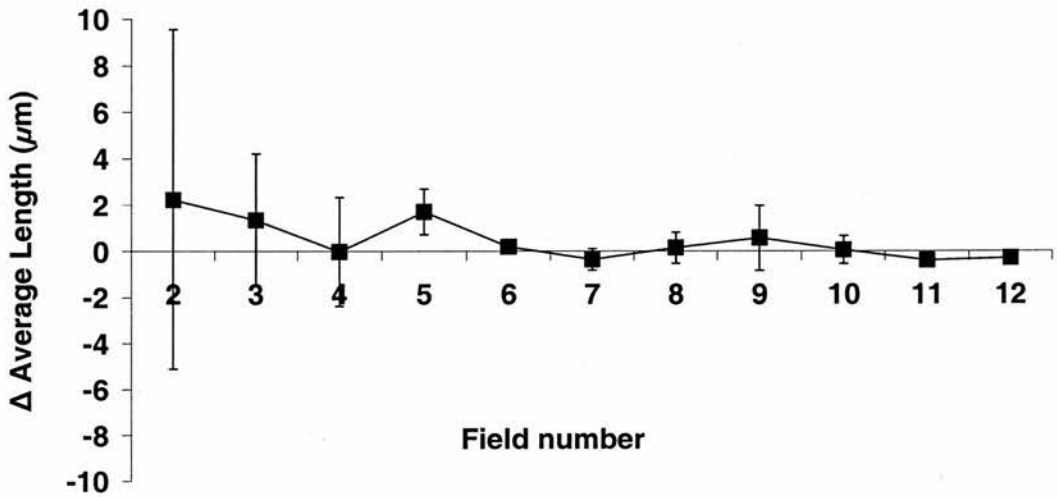


Figure 3.1.3 Population sampling for neurite growth assay

Population sampling for neurite growth assay. Data are presented as means ±SD (n>3). The number of fields which need to be collected to ensure adequate sampling was determined by measuring the standard deviation in the value for the average neurite length from sequential fields. See text for full explanation of results.

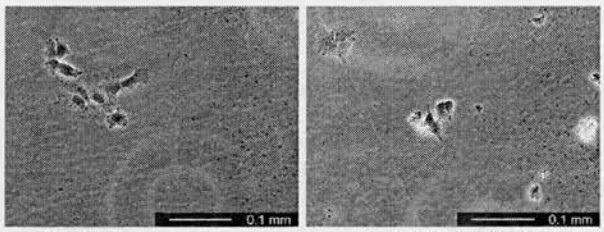
Seeding density

SH-SY5Y cells divide every ~14 hours, thus any experiment lasting more than 24 hours may be hampered by over confluence in the culture dish. We therefore set out to determine the suitable seeding densities. Figure 3.1.4 shows images captured from cells that have been seeded at different densities and left for 24 hours. The images are representative of multiple fields. The pilot study showed that a starting density of 100,000 cells/well led to healthy cells that were not to confluent to allow accurate measurement of neurite length. An NGF treated group was included to ensure that any neurotrophic/differentiating factor would not affect the confluency of the cells.

Incubation times

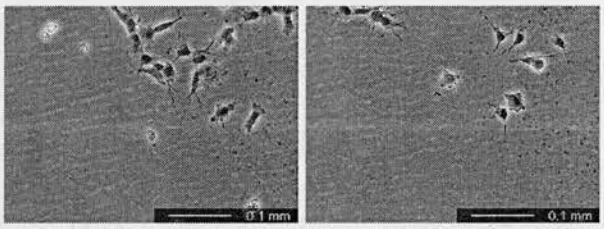
I next determined the suitable time course for the experiment. I wanted to allow the compound I was studying the necessary time to enhance the length of neurites but without leading to over confluent cells. I carried out a pilot study, using NGF as an inducer of neurite growth, that assessed the change in neurite length at 3, 5 and 7 days incubation. The results, shown in Figure 3.1.5, indicate that across three equally powered time points, each representing over 450 neurites only the group at 3 days reached significance (ANOVA, $p<0.05$). In subsequent experiments all drug treatments were for 3 days.

10^3 cell/well



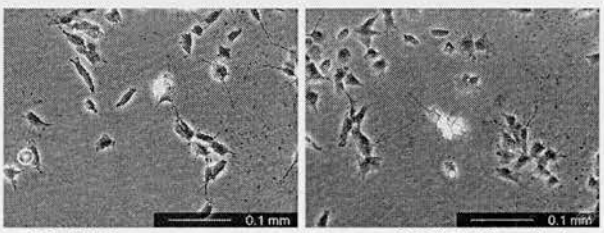
Vehicle NGF 10ng/ml

10^4 cells/well



Vehicle NGF 10ng/ml

10^5 cell/well



Vehicle NGF 10ng/ml

Figure 3.1.4 Seeding densities of SH-SY5Ys

Seeding SH-SY5Ys at a range of densities indicated that 100,000 cells/ well was the best at providing dispersed, healthy, neurite bearing cells. At 1000 cells per well cells would often die and lift off the plate. At 10,000 cells per well, although cell death was much lower, cells were so dispersed that random field sampling often took images with no cells present.

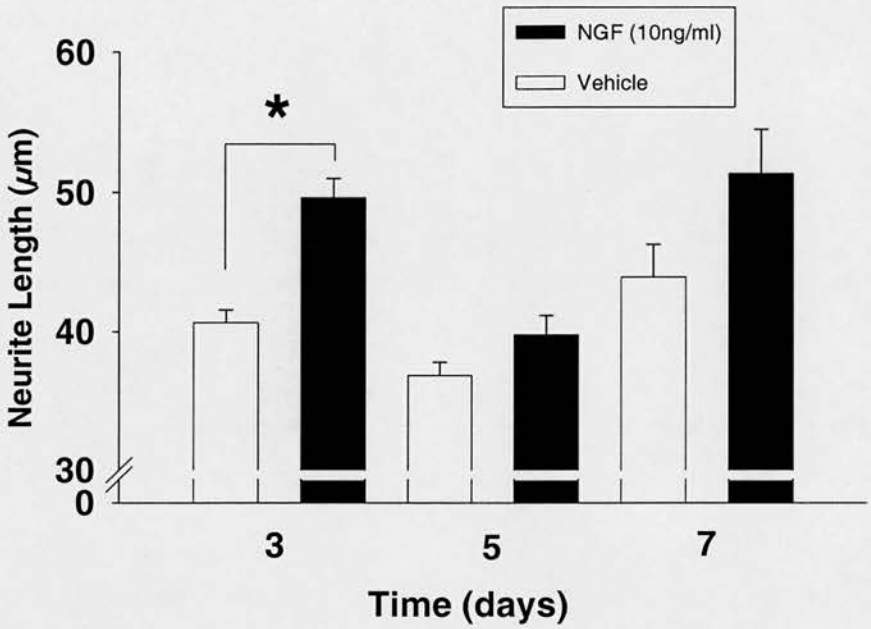


Figure 3.1.5 Incubation times for SH-SY5Ys

Data are presented as mean ±SEM (n>450, p<0.05). Different incubation times were used to determine an appropriate incubation time to assess neurite growth. NGF increased neurite length at each time point but the change was statistically significant only at 3 days with groups of equal size.

3.1.3 The effect of FK506 on neurite growth

To test our model we carried out a large, fully powered study to measure changes in average neurite length following incubation of SH-SY5Y cells with FK506.

FK-506 (tacromilus) is an immunosuppressant used in the prevention of allograft rejection in transplants of organs such as livers and kidneys (Gold 1997). It is also active in the nervous system and has been shown to induce neurite outgrowth, via the activation of MAPK pathways, and potentiate the actions of NGF (Price, Yamaji et al. 2003). Its established neurotrophic role and ready availability made it an ideal compound with which to test the SH-SY5Y neurite growth model.

Treatment of SH-SY5Y cells with FK506 for 72 hrs induced a concentration dependent increase in average neurite length (Figure 3.1.6) with concentrations of 1μM and 10μM producing statistically significant increases compared to vehicle treated cells. Due to the large number of neurites scatter graphs (Figure 3.1.6), although representing the entire data set, are not best at conveying the shift in average length. For this reason data from subsequent studies are presented as mean neurite lengths.

Figure 3.1.7 shows frequency distribution and cumulative frequency graphs to illustrate alternative ways of presenting the large data set. These graphs, presenting the same data as graphs in Figures 3.1.6, reveal a rightward shift in the distribution of neurites across the whole population, suggesting that FK506 promotes the growth of neurites of all lengths. These modes of presenting the data are useful as they reveal additional information about the changes in neurite distribution that may have biological relevance.

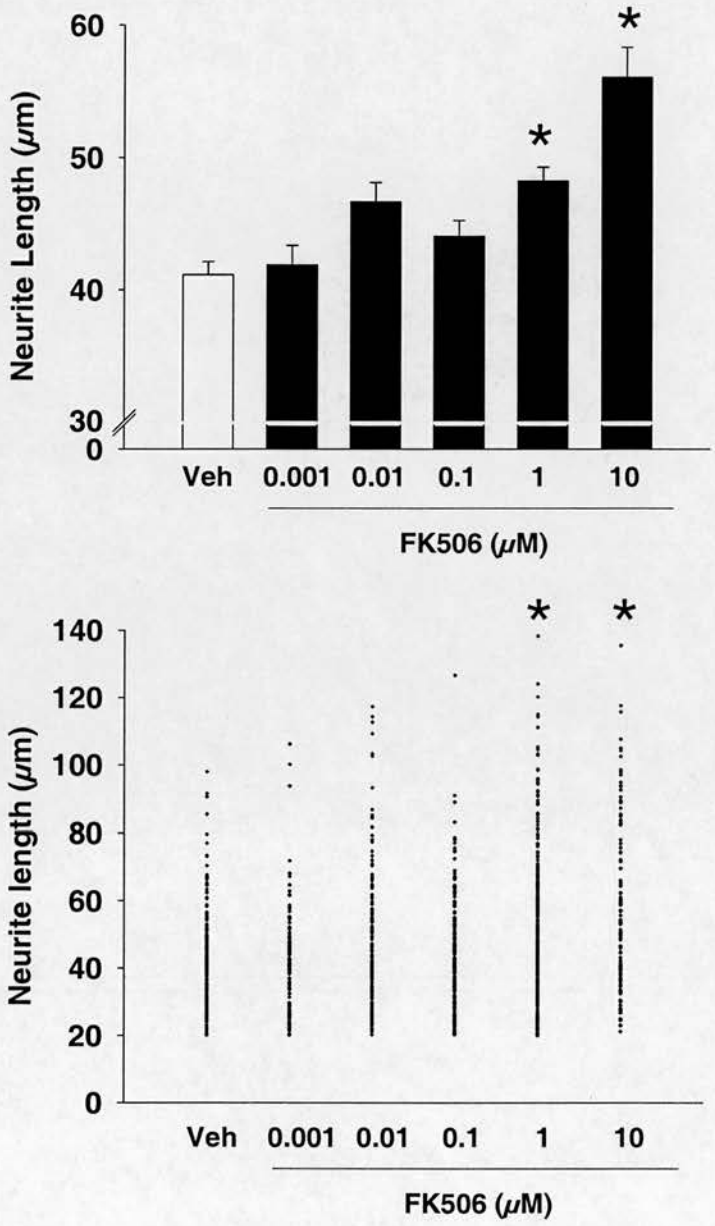


Figure 3.1.6 The effect on neurite growth of FK506

FK506 induces a concentration dependent increase in the average neurite length in SH-SY5Y cells following 72 hours incubation. The same data are presented as mean neurite length ±SEM (upper) and as a scatter graph (lower). veh= vehicle. n>350, p<0.05.

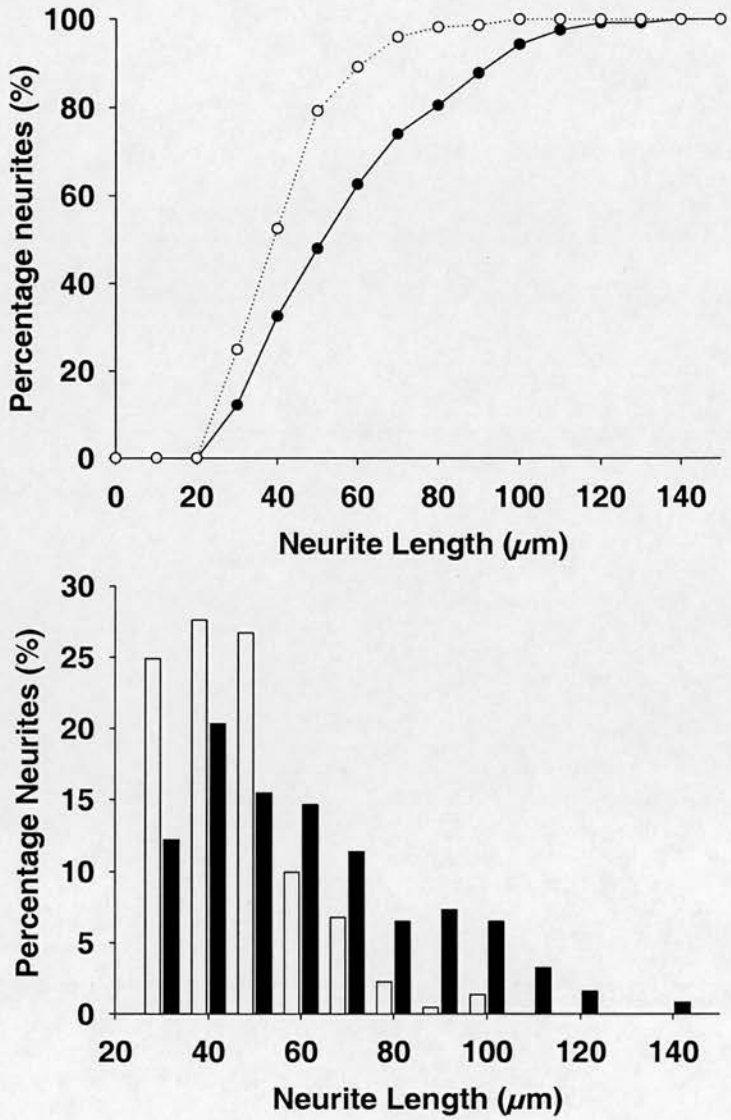


Figure 3.1.7 Frequency distribution of SH-SY5Ys treated with FK506

Frequency distribution graphs of neurites from SH-SY5Ys cells treated with FK506 1μM. Both graphs reveal a rightward shift in the distribution of neurites from FK506 treated cells. Open circles and bars = vehicle, Closed circles bars = FK506 1μM (n>350, p<0.05)

3.1.4 The effect of LY404187 on neurite growth

Following confirmation of the model's sensitivity and robustness the effects of AMPA receptor potentiator LY404187 on neurite length were investigated.

AMPA alone does not enhance neurite growth in SH-SY5Y cells

Application of s-AMPA (the active stearic isomer of AMPA) alone did not enhance neurite length at any concentration tested (Figure 3.1.8) (ANOVA, $p>0.05$). At the highest concentration tested the average length was dramatically accompanied by widespread cell death. The toxicity was due to the actions of s-AMPA as no effect was seen in the vehicle treated cells (1:1000 PBS). As well as excluding any neurotrophic actions of AMPA alone the results also suggest that SH-SY5Ys expressed functional glutamate receptors which mediated the excitotoxic insult (Choi 1992).

LY404187 alone does not enhance neurite growth in SH-SY5Y cells

Administration of LY404187 alone also produced no increase in average neurite length. At the highest concentration tested the compound caused a decrease in the average neurite length (ANOVA, $p>0.05$) (Figure 3.1.9). There was no apparent increase in cell death at this concentration and these observations may reflect some cross reactivity with other receptor subtypes.

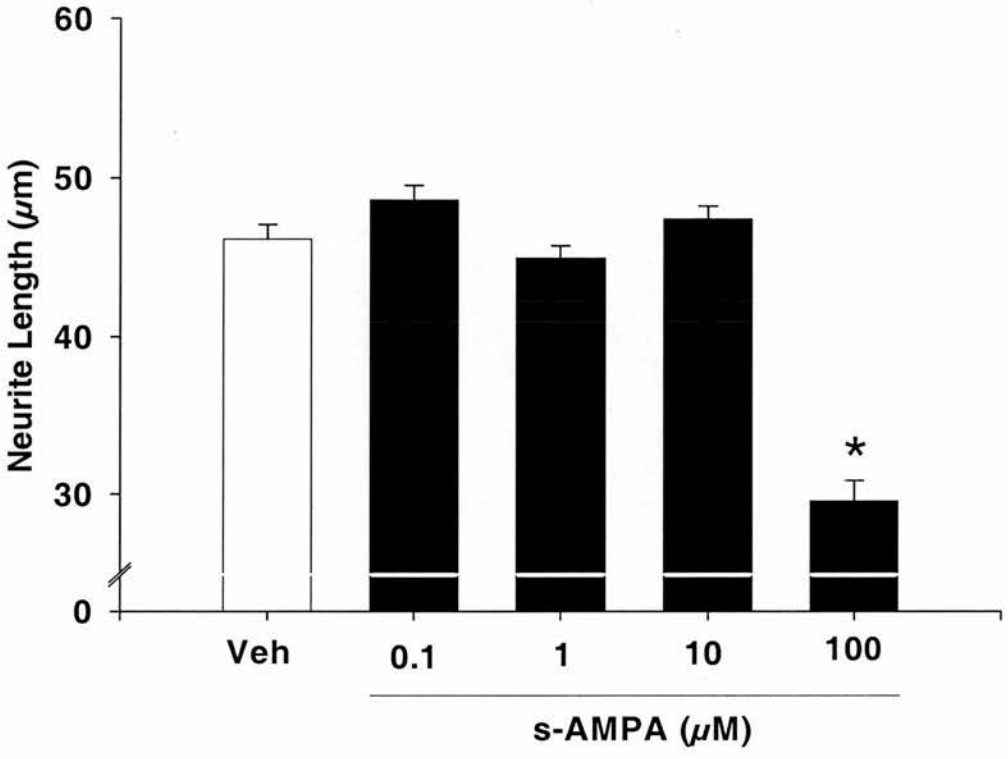


Figure 3.1.8 The effect on neurite growth of s-AMPA

Data are presented as mean ±SEM (n>350, p<0.05). s-AMPA does not promote neurite growth at any of the concentrations tested. 100μM for 72 hours led to widespread cell death and a reduction in the number of neurite bearing cells. Veh= vehicle

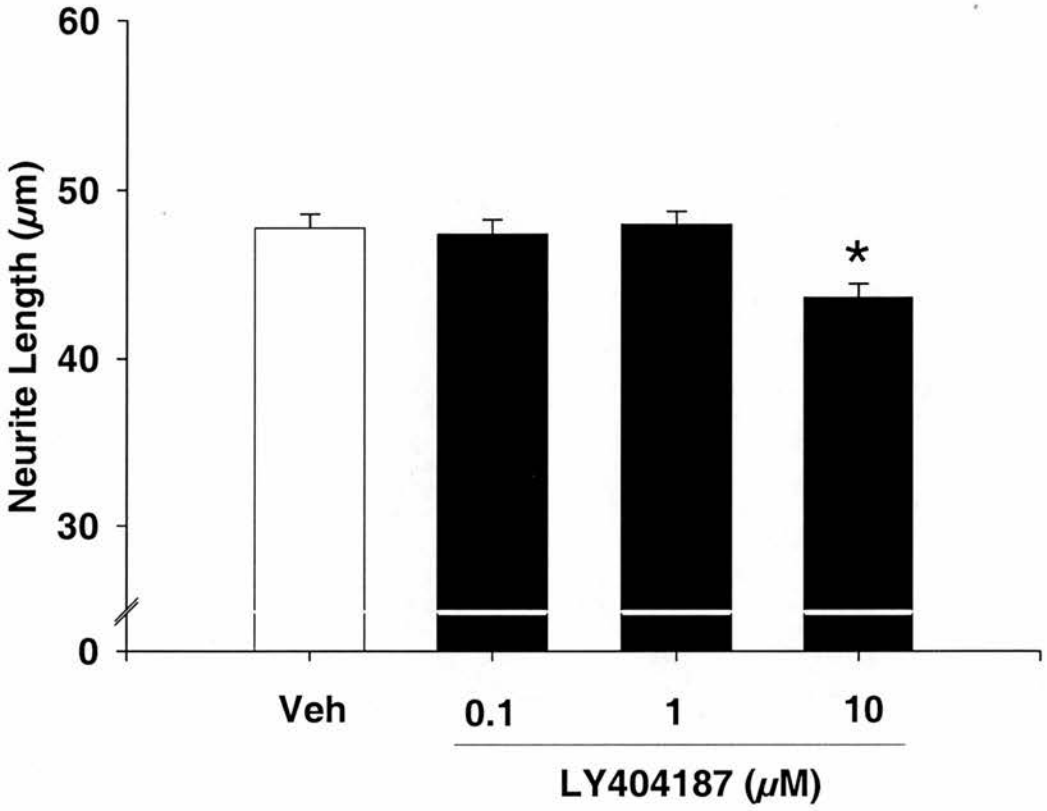


Figure 3.1.9 The effect on neurite growth of LY404187

Data are presented as mean ±SEM (n>350, p<0.05). LY404187 does not promote neurite growth at any concentrations tested. 10μM for 72 hours led to a significant reduction in neurite length. Veh= vehicle

LY404187 in the presence of s-AMPA induces neurite growth

LY404187, in the presence of s-AMPA, when administered at concentrations which alone do not induce neurite growth (0.1 and 1μM), led to a significant increase in neurite length (Figure 3.1.10) (ANOVA, $p<0.05$). Frequency distribution analysis reveals that the increase in average neurite length, although numerically subtle (<10%) represents a pronounced rightward shift in the distribution of shorter neurites (<80μm) (Figure 3.1.11).

Neurite growth induced by LY404187 in attenuated by CNQX

Co-incubation with the AMPA receptor antagonist CNQX (10μM) attenuated the enhancement in average neurite length induced by LY404187 (Figure 3.1.12) (ANOVA with Bonferroni post hoc. test, $p<0.05$). CNQX (10μM) did not significantly alter the average length of cells in the absence of LY404187 and s-AMPA (ANOVA with Bonferroni post hoc. test, $p>0.05$).

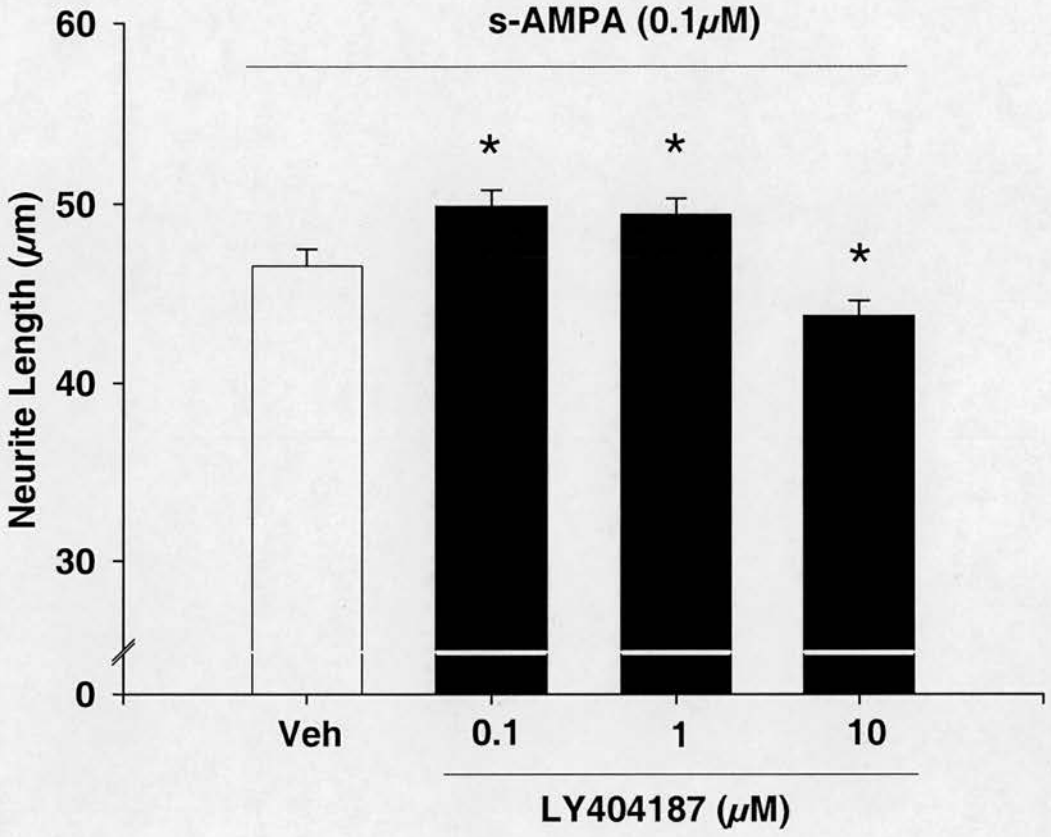


Figure 3.1.10 The effect on neurite growth of LY404187 in the presence of s-AMPA

Data are presented as mean ±SEM (n>350, p<0.05). Co-administration of s-AMPA (0.1μM) and the AMPA potentiator LY404187 (0.1-1μM) for 72hrs significantly increased average neurite length. Veh= vehicle

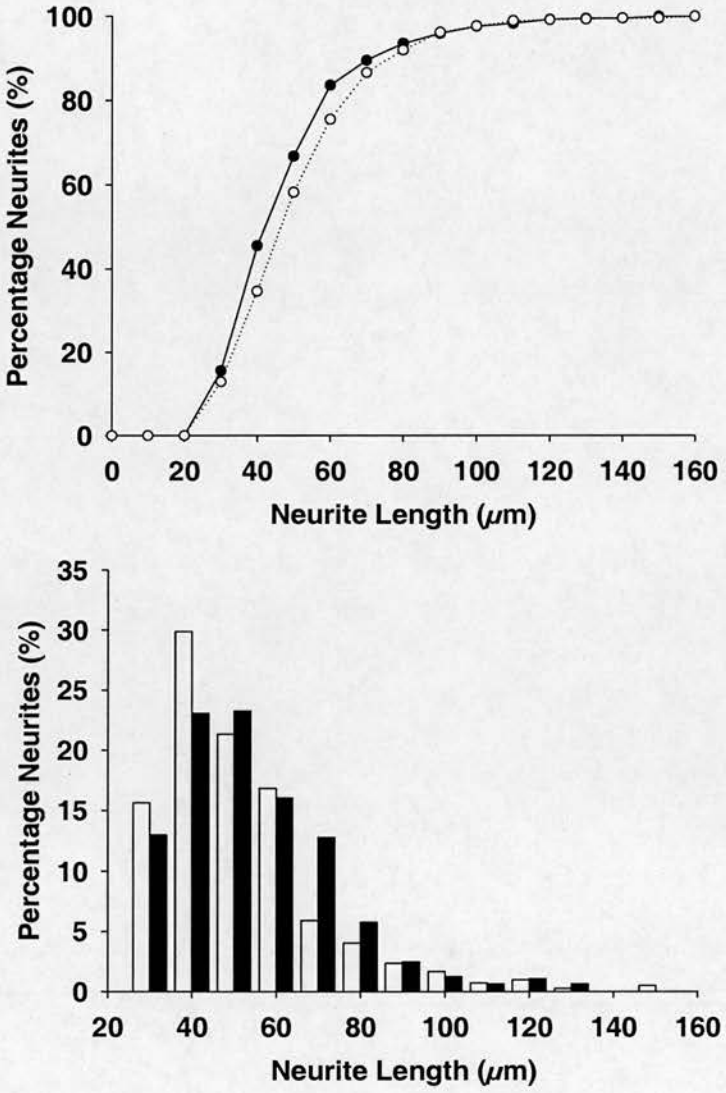


Figure 3.1.11 Frequency distribution of SH-SY5Ys treated with LY404187

Frequency distribution graphs of neurites from SH-SY5Ys cells treated with LY404187 (0.1μM) and sAMPA (0.1μM). Both graphs reveal a rightward shift in the distribution of neurites from LY404187 and s-AMPA treated cells. Open circles and bars = vehicle, Closed circles and bars = LY404187 (0.1μM) and s-AMPA (0.1μM) ($n>350$, $p<0.05$).

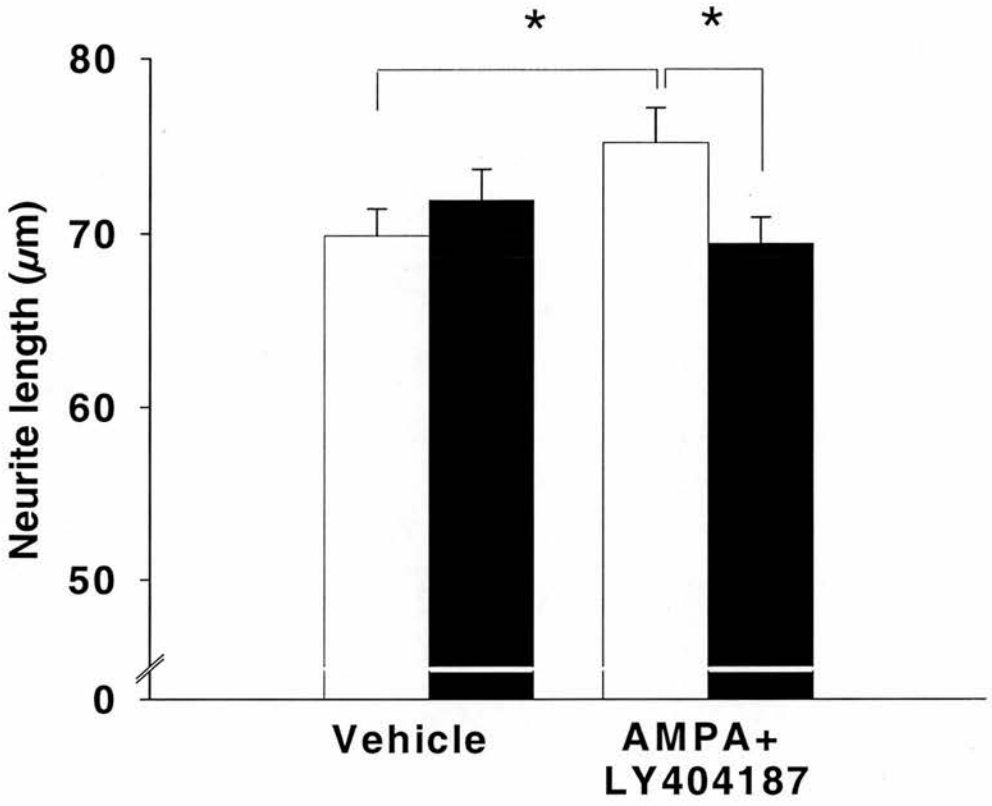


Figure 3.1.12 The effect of CNQX on LY404187-induced neurite growth

Data presented as mean ±SEM (n>350, p<0.05). Incubation of SH-SY5Y with CNQX (10μM) attenuated the increase in neurite length following co-incubation with s-AMPA (0.1μM) and LY404187 (0.1μM). Open bars= vehicle, Closed bars = CNQX (10μM)

3.1.5 The effect of LY404187 on the expression of neurofilament protein

Along with a morphological assessment we also performed biochemical analysis of the effect of LY404187 on SH-SY5Y cells (Figure 3.1.13). Western blot analysis for the cytoskeletal protein neurofilament-heavy chain (NF-H) revealed a similar pattern of response in SH-SY5Ys to that measured by neurite growth. s-AMPA alone failed to enhance NF-H immunoreactivity, as measured by the intensity of a 200kDa band, the predominant band in the blot. LY404187 in the presence of s-AMPA consistently enhanced NF-H in four separate replicate experiments. Although in all studies co-administration of LY404187 and s-AMPA induced the largest increase in NF-H a less reproducible but evident increase was seen with administration of LY404187 alone. In all treatment groups NF-H immunoreactivity was decreased by co-incubation with CNQX (10μM). Equal loading was confirmed by the relative intensity of actin blots across wells (42kDa band).

Taken together with the structural data these results indicated that AMPA receptor potentiation promotes significant cytoskeletal development.

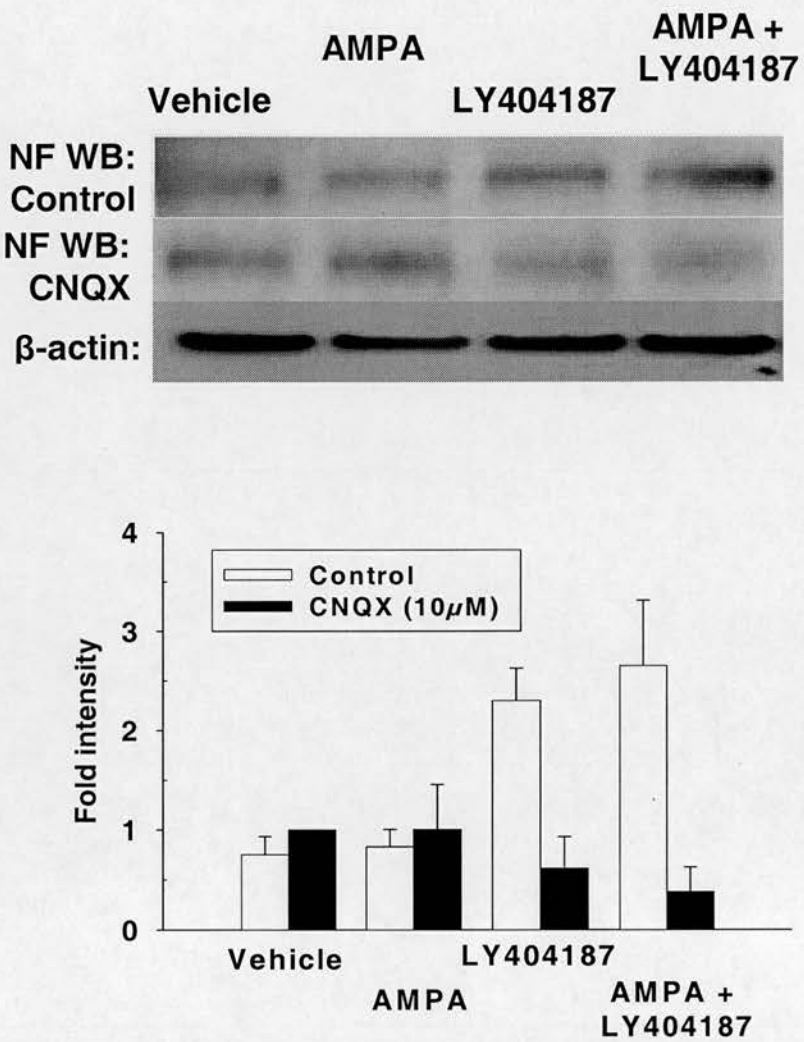


Figure 3.1.13 The effect of LY404187 on neurofilament expression

Data are presented as mean ±SEM fold increase in chemiluminescence normalised to Vehicle+CNQX treated cells, (n=4). Representative Western blots showing neurofilament heavy chain expression levels from control (upper) and CNQX (10μM) treated cells (middle). Equal loading was confirmed by blotting for β-actin (lower).

3.1.6 The comparative effects of multiple compounds on neurite growth

Because I had established a new method for the measurement of neurite length *in vitro* we carried out a study comparing the magnitude of increase in neurite length seen following incubation with LY404187 in the presence of s-AMPA with that of other well characterised compounds. The neurotrophic factors BDNF and NGF as well as the differentiating factor RA were used to induce neurite length in SH-SY5Ys. The concentrations used were chosen from larger studies using these compounds carried out in parallel to the studies described in this document (Milne and Voss unpublished observations). Each compound was dissolved in a different vehicle (DMSO, ethanol, glycerol and PBS). I therefore carried out independent, concurrent and equally powered experiments to compare the magnitude of the effects of each compound. Figure 3.1.14 shows the results from these studies. The data were analysed by Student t-tests on the average lengths from each experiment. The data indicate that NGF and RA induce a 30% increase in average neurite length while BDNF and LY404187 in the presence of s-AMPA enhance neurite length by approximately 10%.

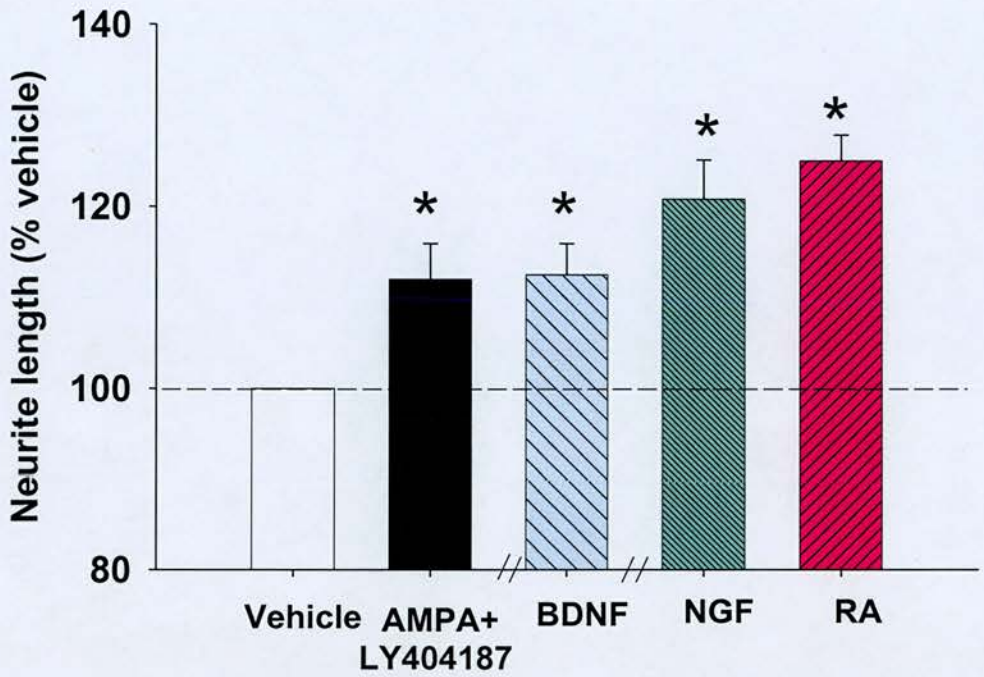


Figure 3.1.14 Comparison of neurite growth induced by LY404187, NGF, BDNF and RA

Data presented as mean ±SEM neurite length (n>400, p<0.05). Increases in neurite length following BDNF (10ng/ml), NGF (10ng/ml) and retinoic acid (RA) (0.1μM) treatment are compared to AMPA (0.1μM) + LY404187 (0.1μM) (A+LY) treated cells.

3.1.7 The effect of LY404187 on the expression of Trk receptor protein

AMPA receptor potentiators have been shown to increase expression of BDNF mRNA and protein *in vitro* (Lauterborn, Lynch et al. 2000; Legutko, Li et al. 2001) and *in vivo* (Mackowiak, O'Neill et al. 2002). Intracellular actions of BDNF are mediated by binding to Trk-B receptors. Un-differentiated SH-SY5Y cells express low levels of Trk-B receptor protein and increase their expression significantly following differentiation (Ruiz-Leon and Pascual 2003).

We therefore investigate the role of Trk receptors in the effect of LY404187 on SH-SY5Y cells. Levels of Trk receptor protein were measured by Western blotting. LY404187 in the presence of s-AMPA consistently enhanced Trk expression in four separate replicate experiments, as measured by the intensity of a 140kDa band (Figure 3.1.15). Incubation with s-AMPA or LY404187 alone produced no increase in Trk immunoreactivity. Equal loading was confirmed by the relative intensity of actin blots across wells (42kDa band). The increase in Trk levels could be blocked with the addition of the AMPA receptor antagonist CNQX (10μM).

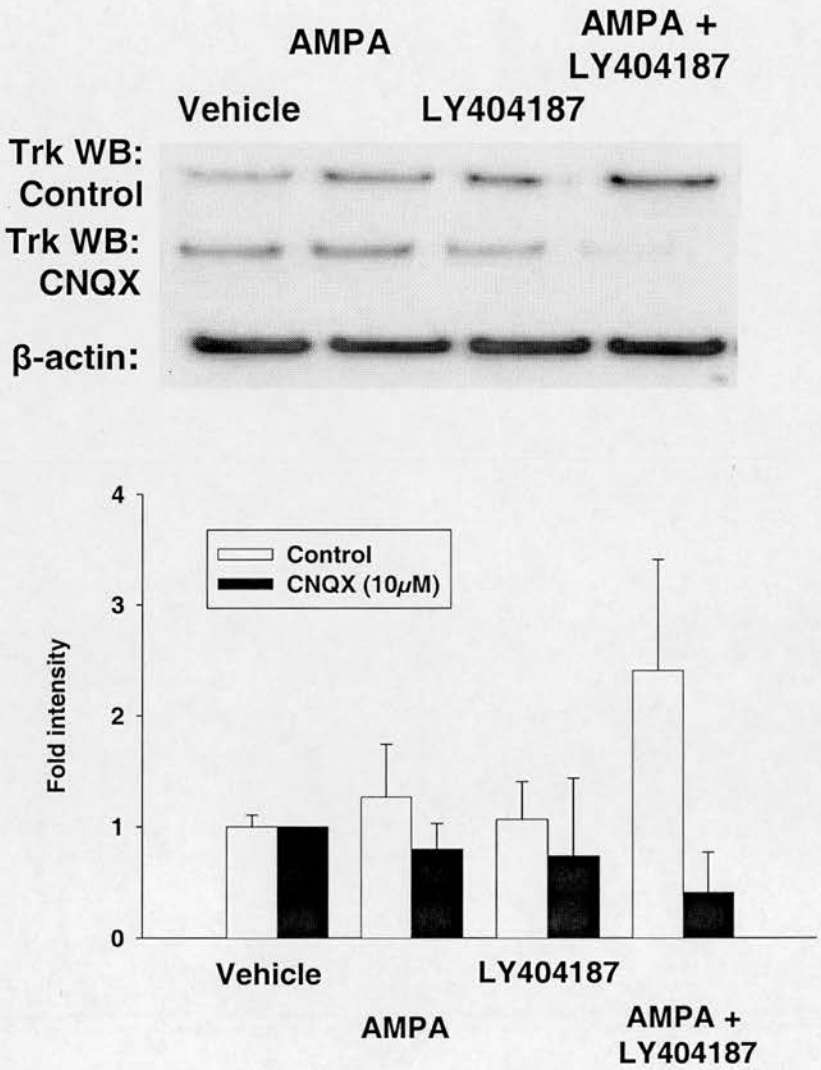


Figure 3.1.15 The effect of LY404187 on Trk receptor expression

Data are presented as mean ±SEM fold increase in chemiluminescence normalised to Vehicle+CNQX treated cells, (n=4). Representative Western blots showing Trk receptor expression levels from control (upper) and CNQX (10μM) treated cells (middle). Equal loading was confirmed by blotting for β-actin (lower).

3.1.8 The effect of anti-BDNF IgG on LY404187-induced neurite growth

Increased Trk receptor expression suggests neurotrophin function is important in this model and the role of BDNF on neurite growth was thus examined. Attempts to monitor levels of BDNF protein by Western Blot and ELISA assays were unsuccessful (data not shown). Evidence from the literature however indicates that SH-SY5Ys expressed very low levels of BDNF protein (Olivieri, Otten et al. 2003).

Antibody sequestration is a common technique to investigate the physiological role of neurotrophins (Barde, Edgar et al. 1982; Cohen-Cory and Fraser 1995; Tucker, Meyer et al. 2001). The addition of an antibody against BDNF inhibited the increase in neurite length induce by LY404187 and s-AMPA (ANOVA with Bonferroni post hoc test, $p<0.05$). The antibody did not significantly alter the average neurite length of any other treatment group (Figure 3.1.16) and control experiments were carried out to determine the specificity of the BDNF antibody. Western blots identified a single band at 14 kDa corresponding to BDNF (Figure 3.1.17). The growth of neurites over 72 hours was unaltered following incubation of BDNF antibody and, crucially the addition of a random rabbit IgG did not attenuate the increase in neurite length following the co-incubation of LY404187 and s-AMPA These data implicate BDNF in the mechanisms of the neurite growth promoted by AMPA receptor potentiation.

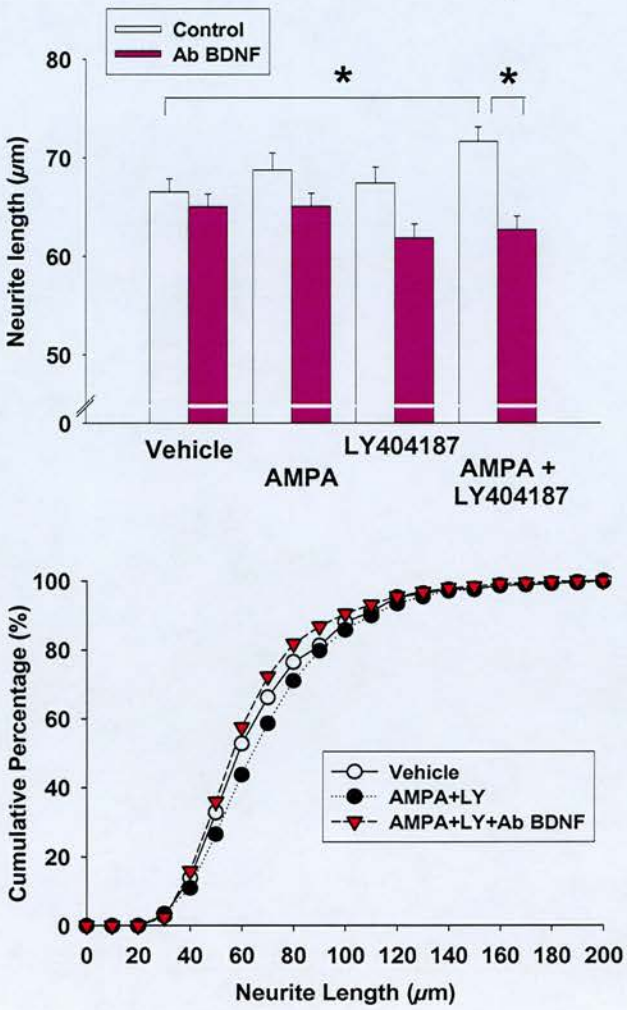


Figure 3.1.16 The effect anti-BDNF IgG on LY404187-induced neurite growth

(Upper) Data are presented as mean ±SEM neurite lengths. (n>450, p<0.05). (Lower) The same data are presented as cumulative percentage (n>450, p<0.05). Co-incubation with anti-BDNF mouse IgG (Ab BDNF) (1μg/ml) attenuated neurite outgrowth following co-incubation with s-AMPA (0.1μM) and LY404187 (0.1μM).

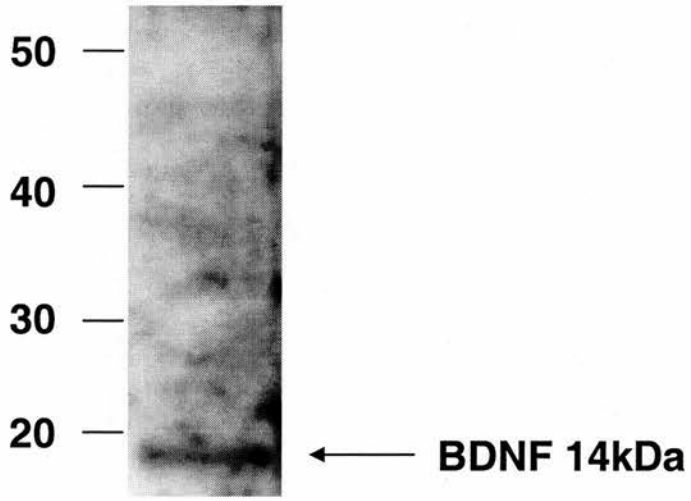


Figure 3.1.17 Western blot showing specificity of anti-BDNF antibody

Western blot showing BDNF (14kDa) in untreated SH-SY5Y lysate. The specificity of the antibody is evident as a single clear band was detected.

3.2 The effect of LY404187 on neurite sprouting and neurogenesis in the dentate gyrus of mice following unilateral entorhinal cortex lesions

3.2.1 Aims

The aim of this study was to establish a model of structural plasticity *in vivo* in which to measure the effect of LY404187 neuronal sprouting. To this end I used unilateral lesions of the entorhinal cortex which induce unilateral loss of synapses in the molecular layer of the dentate gyrus (DG) of the hippocampus. Over time sprouting from within the hippocampal formation leads to a recovery in the density of synapses in the molecular layer.

3.2.2 Establishment of mouse model of ECL

To determine the appropriate time points at which to investigate the effects of LY404187 on sprouting in the hippocampus a time course of the loss and recovery of synaptophysin in the middle molecular layer of the DG in mice was performed. Using the study by White et al. (2001) as a guide I analysed the ratio of optical densities between hemispheres at 3, 7, 28 and 90 days post surgery.

Unilateral excitotoxic lesions of the entorhinal cortex failed to produce a significant change in the ratio of ipsilateral and contralateral synaptophysin levels in the middle molecular layer of the dentate gyrus. However there was a significant difference between the average interhemispheric ratio at 3 and 7 days post surgery (Figures 3.2.1 and 3.2.2) (ANOVA, $p<0.05$). Interhemispheric ratio of synaptophysin optical densities: 3 days 0.995 ± 0.028 and 7 days 0.899 ± 0.059 $p<0.05$). No differences were seen between

other time groups. This apparent contradiction with other published studies (White et al. 2001) is most likely due to problems with the dose of ibotenic acid used to produce the lesions. In fact, although histological assessment of the lesion site revealed necrotic tissue around the injection site and confirmed the correct placement of the syringe (Figure 3.2.3), most animals in the 28 day recovery group displayed small lesions (data not shown). These animals were all operated on at the same time indicating that a faulty dose of ibotenic acid would have led to reduced lesions in this group. In subsequent experiments animals from different recovery groups were lesioned of different days.

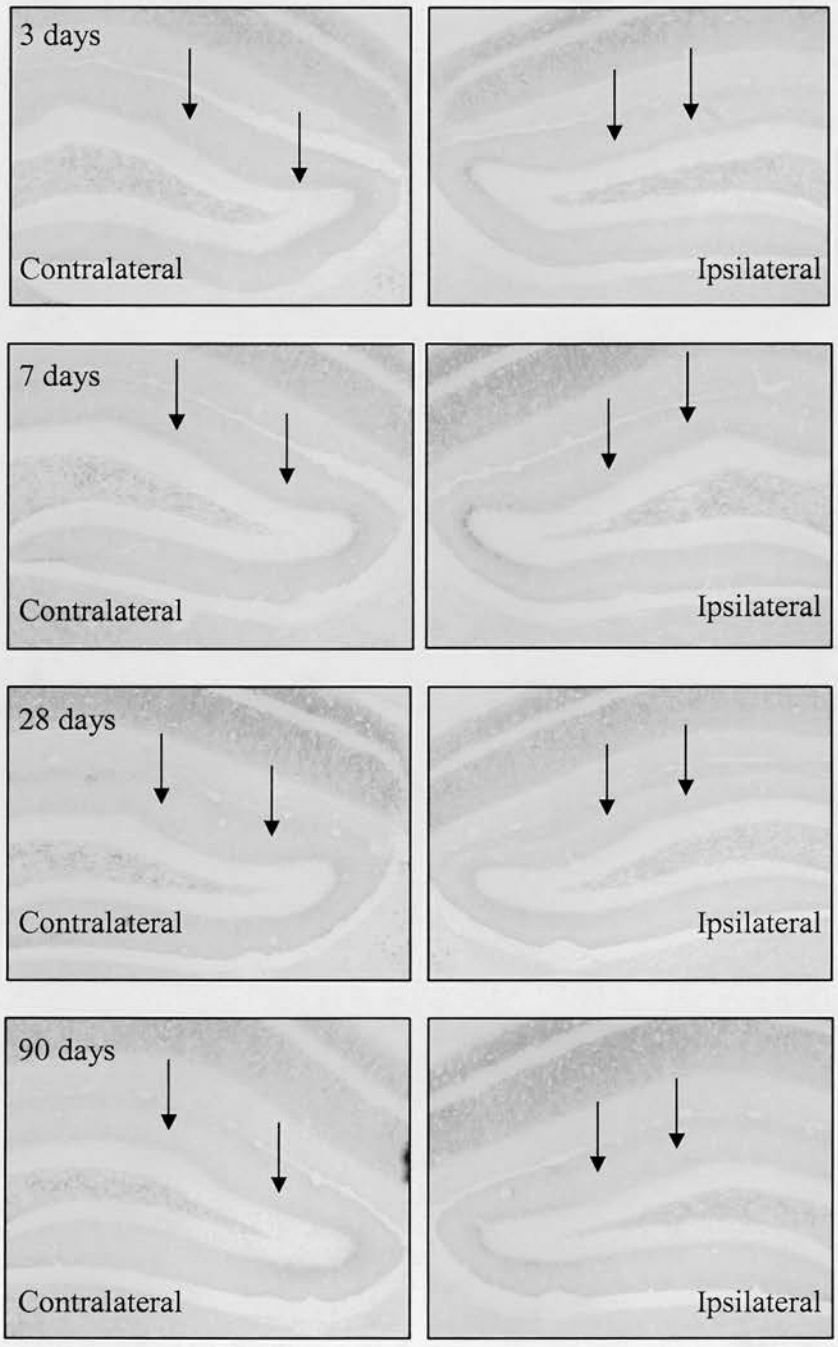


Figure 3.2.1 Synaptophysin immunoreactivity in the dentate gyrus

7 days post-unilateral ECL there is a significant reduction in synaptophysin staining along the middle molecular layer of the dentate gyrus ipsilateral to the lesion site (black arrows)

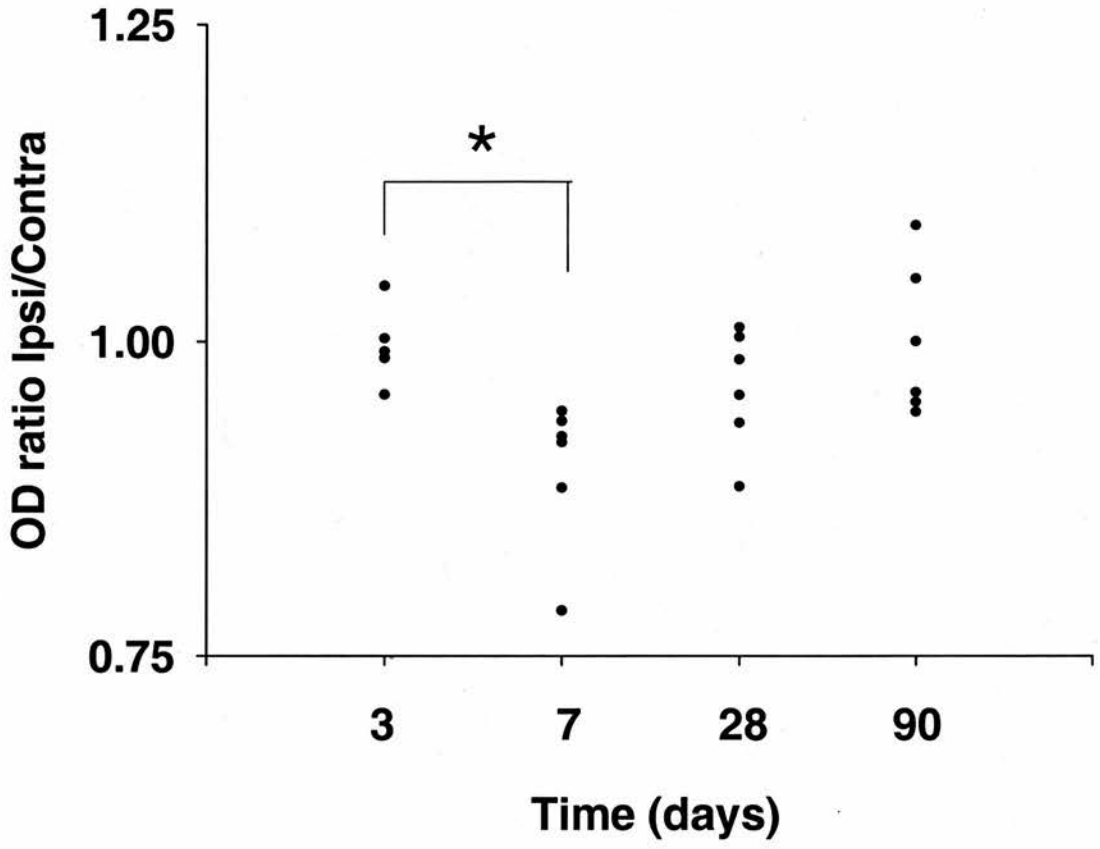


Figure 3.2.2 Ratio of inter-hemispheric synaptophysin levels

Ratios of optical density measurements along the middle molecular layer of the DG in mice that have undergone unilateral ECL (n=6, p<0.05).

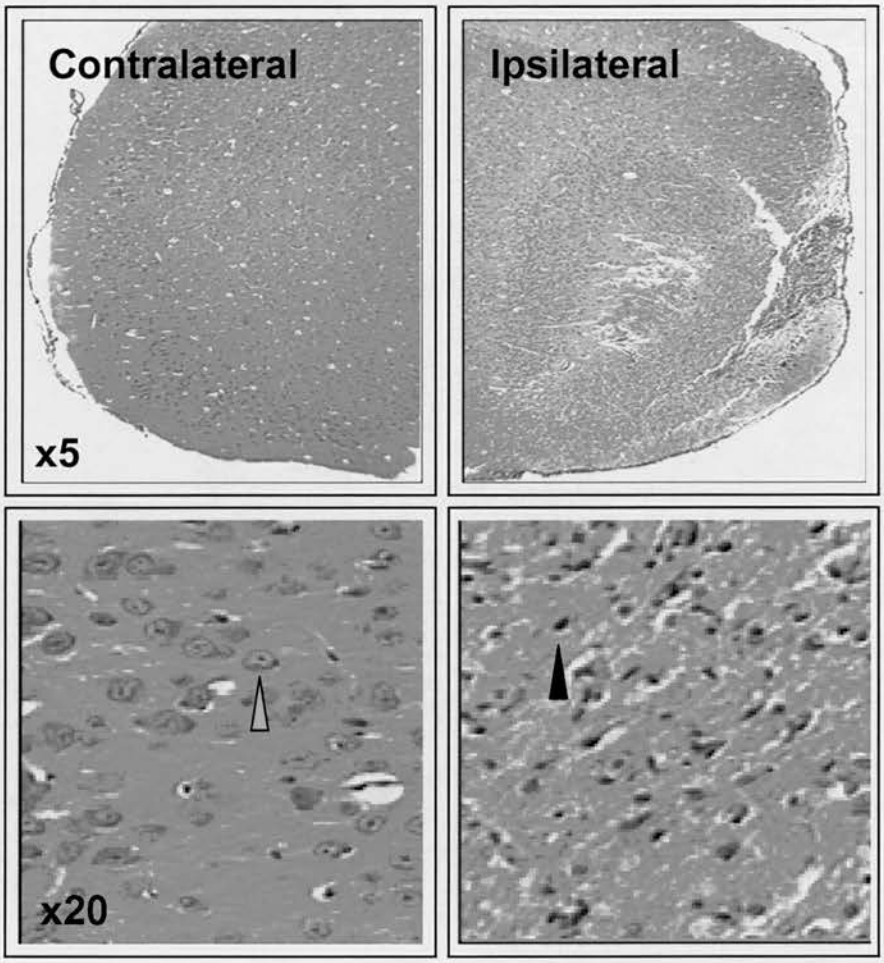


Figure 3.2.3 Histological analysis of lesioned tissue

Photomicrographs showing the damage caused by unilateral ibotenate injections in the entorhinal cortex. Lesioned tissue was pale and contained many densely stained shrunken cell bodies (black arrow). Un-lesioned tissue from the contralateral entorhinal cortex is shown for comparison displaying intact neuropil and large cell bodies (yellow arrow).

3.2.3 The effect of LY404187 on lesion volumes and synaptophysin levels following ECL

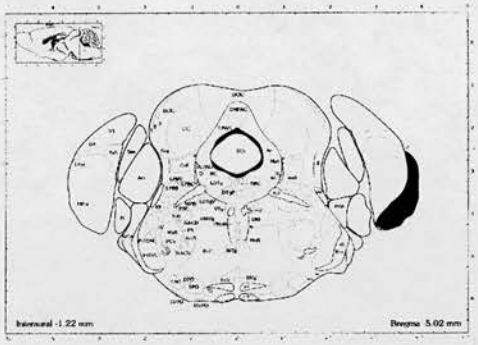
Following the pilot study in which we characterised the progression of synaptic degeneration, I next carried out a new study on the effects of LY404187 reactive sprouting following surgery. We hypothesised that the reactive sprouting in the dentate gyrus would be enhanced following treatment with the AMPA potentiator LY404187. The mice underwent unilateral lesion of the entorhinal cortex and were treated with LY404187 twice daily (0.5 mg/kg s.c) for 14 and 28 days and the lesion size and the extent of synaptophysin levels were measured in both hemispheres of the dentate gyrus.

One animal was excluded from further study due to its lesion extending rostrally into the hippocampal formation (Figures 3.2.4 and 3.2.5). The average lesion volume across all groups was 1.36 mm^3 with maximum and minimum volumes of 2.8 and 0.4 mm^3 (Figure 3.2.6). There was no significant change in average lesion size following administration of LY404187 0.5mg/kg, nor was there any difference between 14 and 28 day groups (ANOVA, with Bonferroni post hoc test, $p>0.05$).

Figure 3.2.4 Representative entorhinal cortex lesions

(overleaf) Lesion template from a representative animal (AR8) and from animal AR44 that was excluded from further study because of damage to the neocortex and hippocampus. Red areas represent lesioned tissue. These areas were used for 3D reconstruction and volume calculations.

AR8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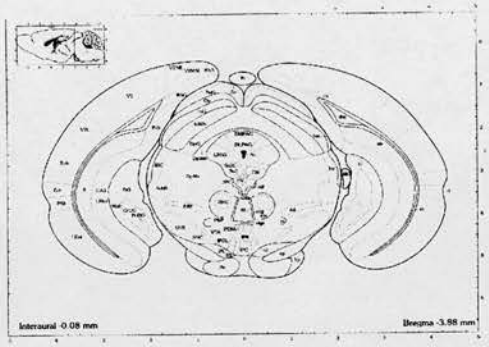
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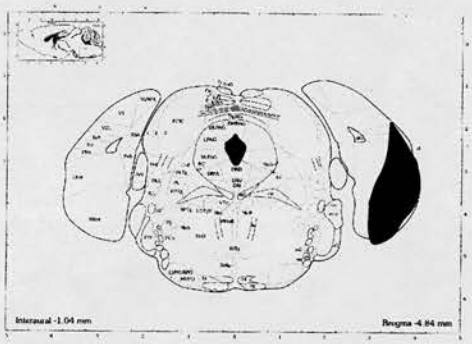
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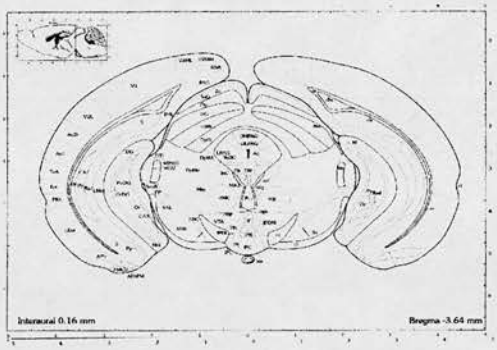
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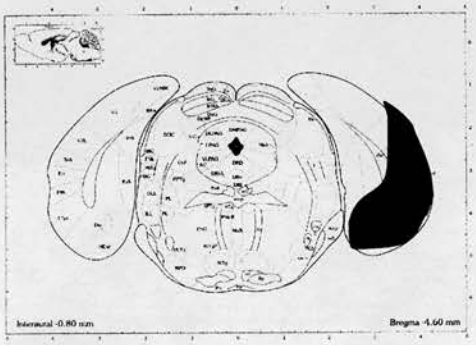
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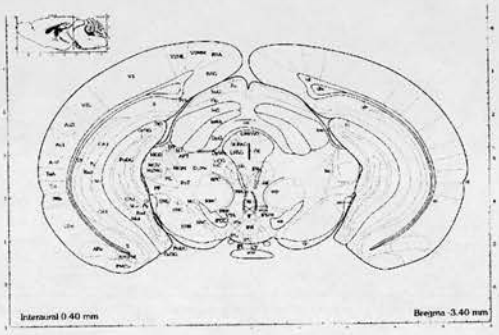
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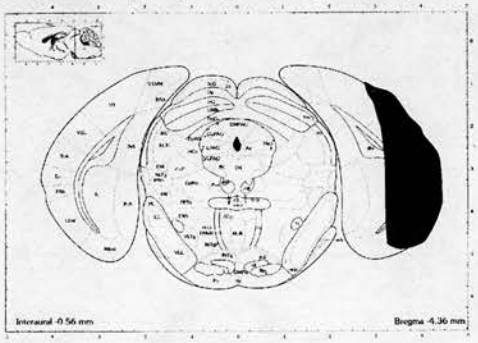
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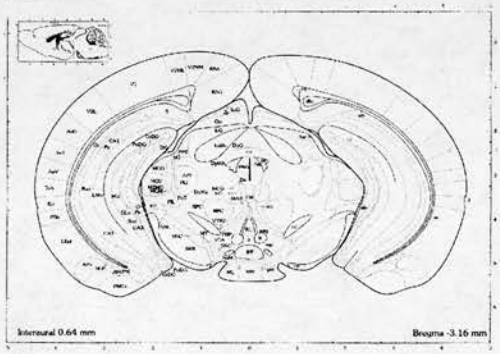
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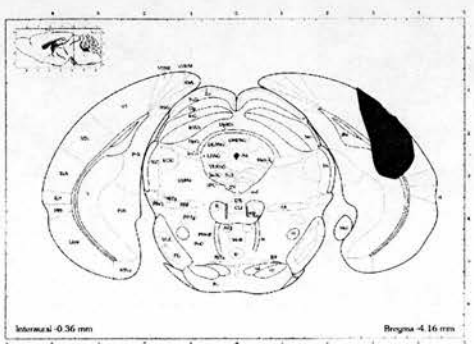
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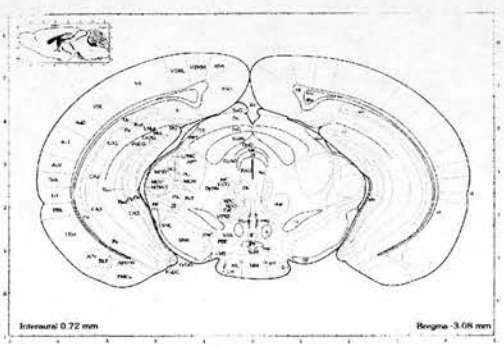
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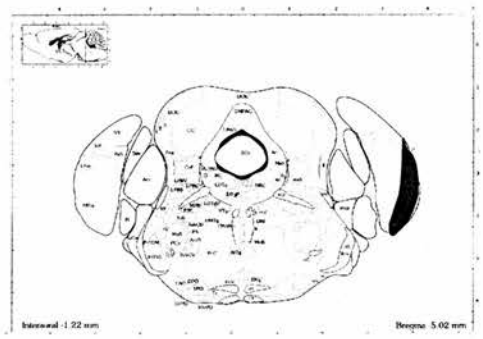
AR44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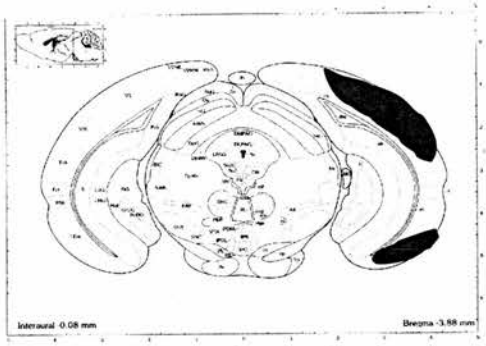
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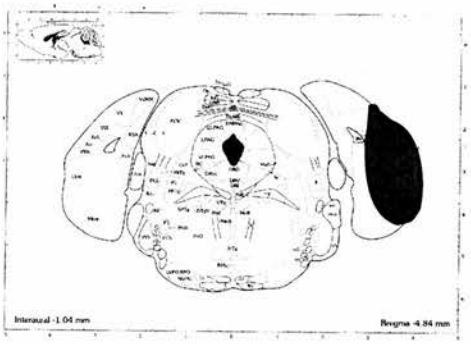
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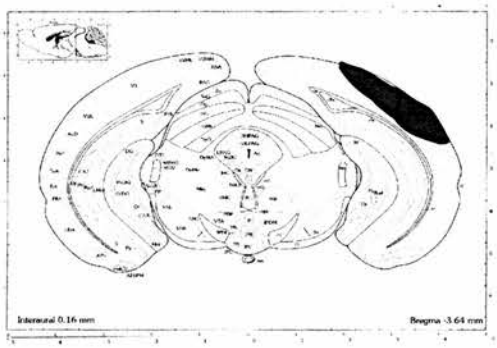
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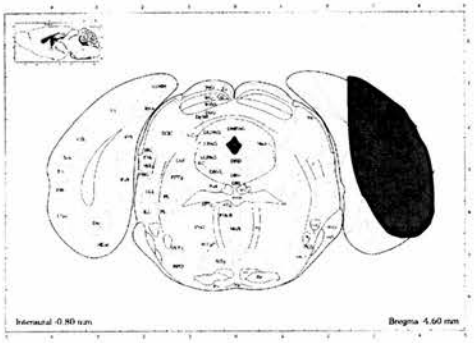
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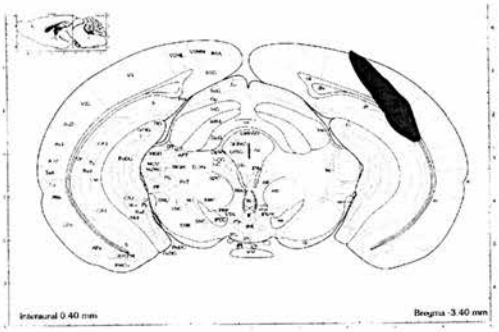
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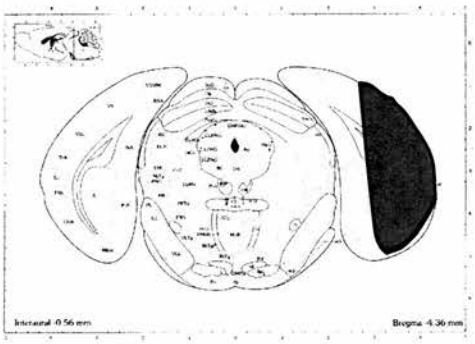
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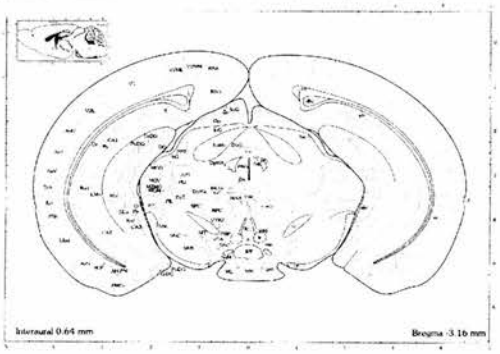
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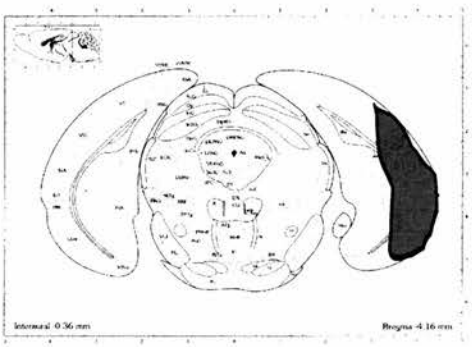
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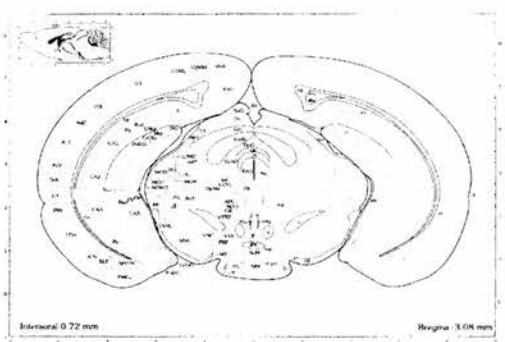
Bregma -4.16 mm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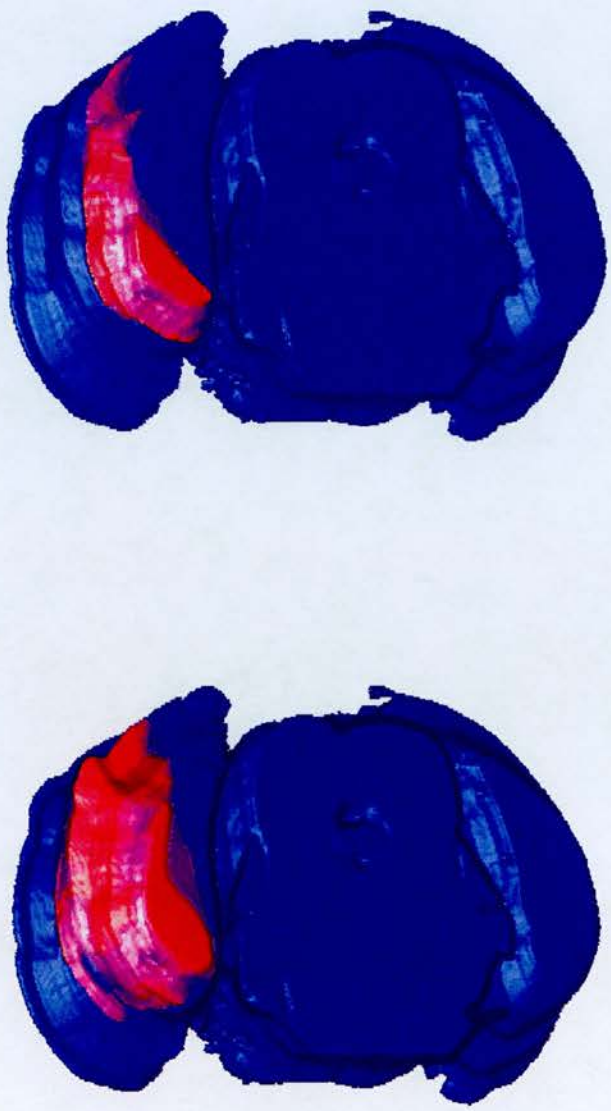


Figure 3.2.5 3-D Reconstructions of representative lesions

3D reconstruction of lesions from animals AR8 (upper) and AR44 (lower). Figure shows lesioned tissue (red) in the posterior 3mm of the entorhinal cortex (blue).

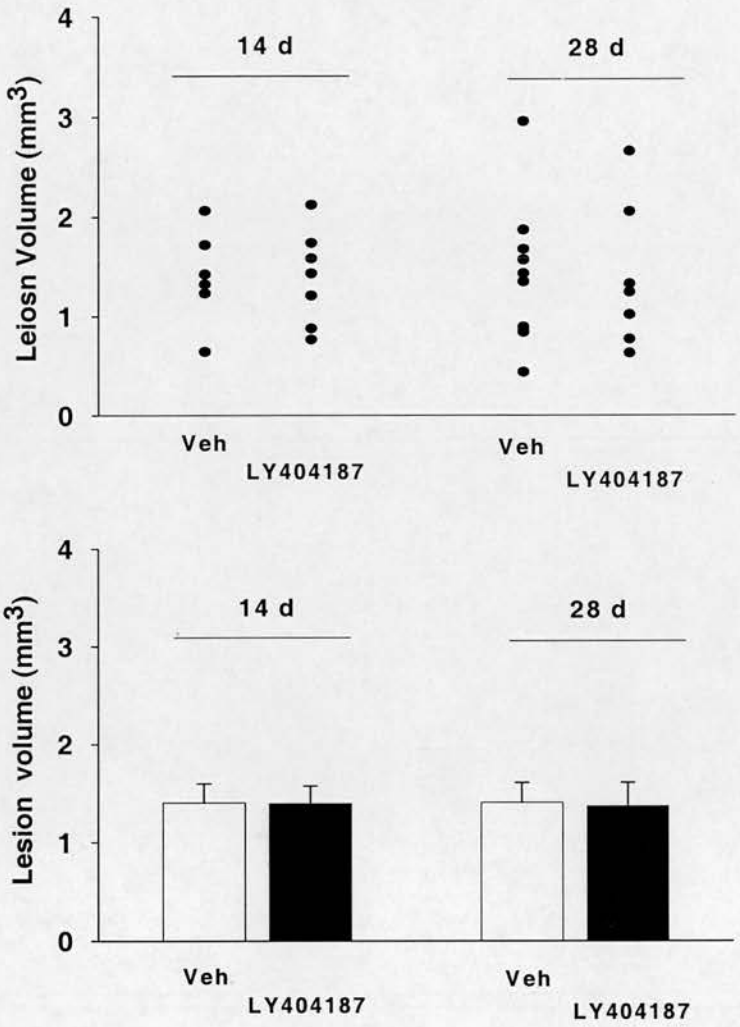


Figure 3.2.6 Lesion volumes in the entorhinal cortex following chronic administration of LY404187

(Upper) Data are presented as a scatter graph of the raw data. One animal (arrow) was excluded from further study because its lesion spread rostrally into the hippocampal formation and the volume was over twice the mean for that group. (Lower) Data are presented as the mean ±SEM volumes for the animals included in further study.

Once the lesions had been quantified and animals included or excluded from the study we measured the primary end point of the study, synaptophysin immunoreactivity in the dentate gyrus. 10 optical density readings were taken from 3 fields across the dentate gyrus; dorsal, medial and ventral. Readings were also taken from the corpus callosum and the average value was subtracted from the average from the dentate gyrus. The experimenter was formally blinded against treatment groups and the codes were broken after all data was collected. Figure 3.2.7 summarises the results of this study. There was no significant difference between the ratio of ipsilateral to contralateral synaptophysin immunoreactivity between animals dosed with LY404187 and animals dosed with vehicle.

Larger lesions cause more profound loss of synapses and a subsequently greater reduction in synaptophysin immunoreactivity in the dentate gyrus (van Groen 2001; van Groen, Miettinen et al. 2003). Correlation analysis between the volume of the lesion and synaptophysin immunoreactivity revealed a weak correlation between the two parameters at 14 days post lesion but no correlation between lesion and synaptophysin levels (Figure 3.2.8). 14days; vehicle r^2= 0.242, LY404187 r^2=0.246. 28 days; vehicle r^2=0.003, LY404187 r^2= 0.009.

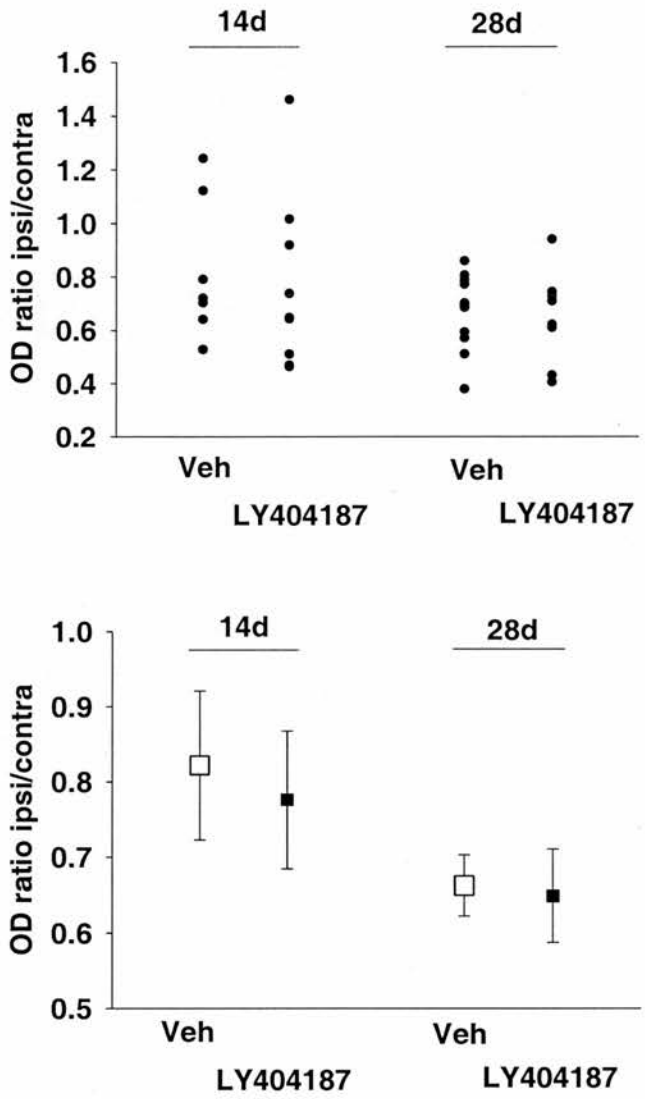


Figure 3.2.7 Synaptophysin levels in the dentate gyrus following chronic administration of LY404187

Synaptophysin labelling in the middle molecular layer of the dentate gyrus. (Upper) data are presented as scatter graph of raw optical density ratios. (Lower) The same data are presented as mean ±SEM (n=7-10).

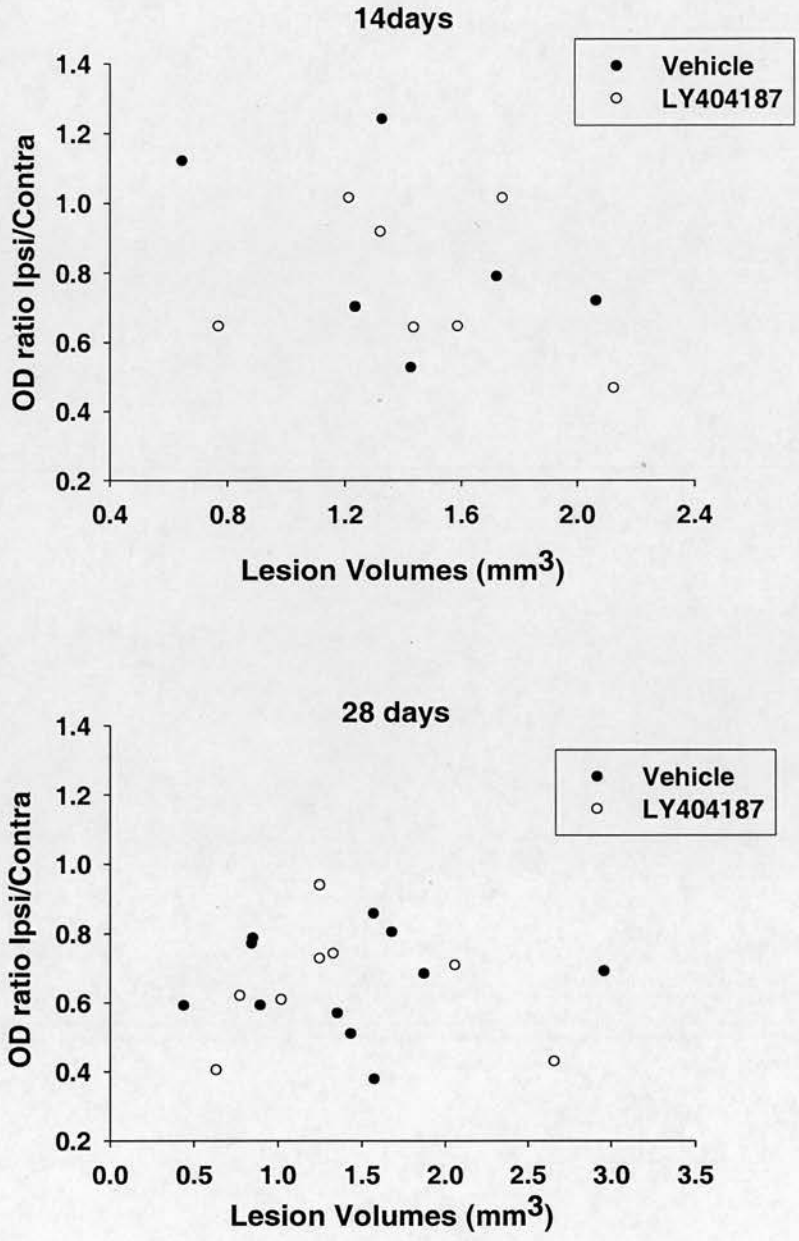


Figure 3.2.8 Correlation of lesion volume and synaptophysin levels

Data are presented as a scatter graph of lesion volume against synaptophysin levels for 14 day (upper) and 28 day (lower) treatment groups.

3.2.4 The effect of LY404187 on the rate of neurogenesis in the dentate gyrus following ECL

The second end point of the study was to monitor the rate of neurogenesis in the dentate gyrus 14 and 28 days following ECL. The study was also aimed at detecting any change induced by the administration of LY404187.

Currently there are multiple protocols to study the rate of neurogenesis in the hippocampal formation. The most widely used method is the administration of the uridine analogue BrdU. The compound is incorporated into newly synthesised DNA and $BrdU^+$ cells can be detected using simple immunological techniques. In the current study I chose to count, in both hemispheres of the dentate gyrus at the level of the lateral habenulae, the average number of BrdU positive cells per section using two adjacent slides.

The detection of $BrdU^+$ cells in the sub-granular region of dentate gyrus of tissue sections with anti-BrdU antibody allowed clear and easily quantifiable detection of newly generated cells (Figure 3.2.9).

The rate of neurogenesis, as measured by the number of cells incorporating BrdU, did not change across time points in ipsilateral or contralateral hemispheres (ANOVA, $p>0.05$). There was no change in the rate of neurogenesis between animals administered LY404187 and animals administered vehicle (Figure 3.2.10) (ANOVA, $p>0.05$). There was also no change in the ratio of ipsilateral to contralateral hemispheres across all groups (Figure 3.2.11) (ANOVA, $p>0.05$).

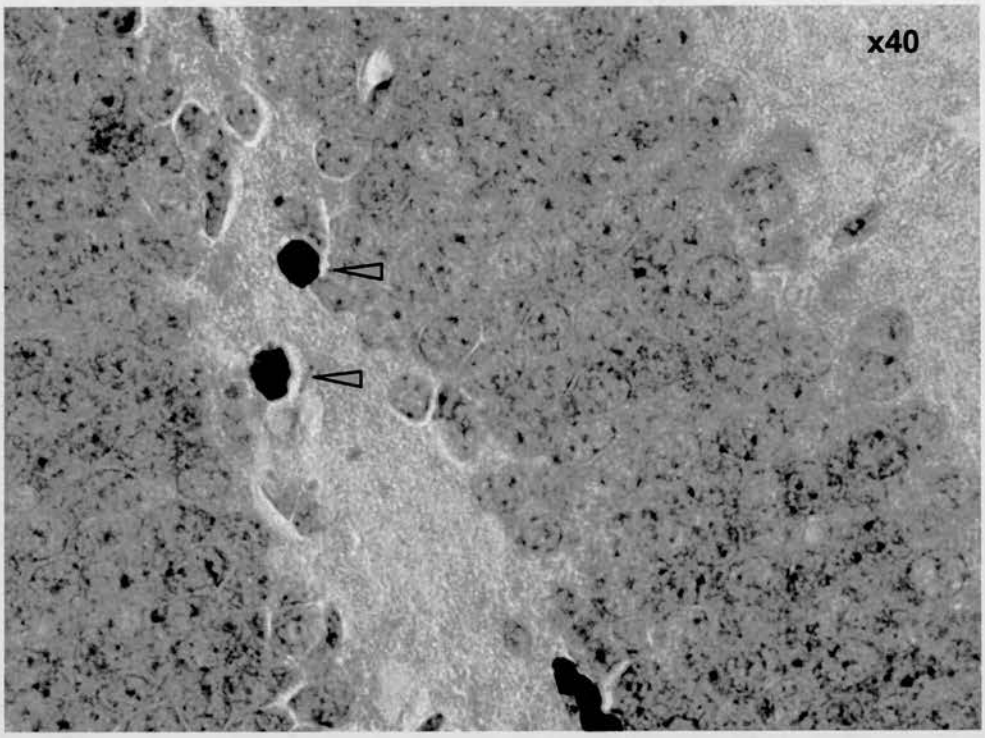


Figure 3.2.9 Detection of neurogenesis in hippocampus

$BrdU^+$ cells in the sub-granular layer of the dentate gyrus (yellow arrows) of normal mice. Tissue is counterstained with heamatoxylin.

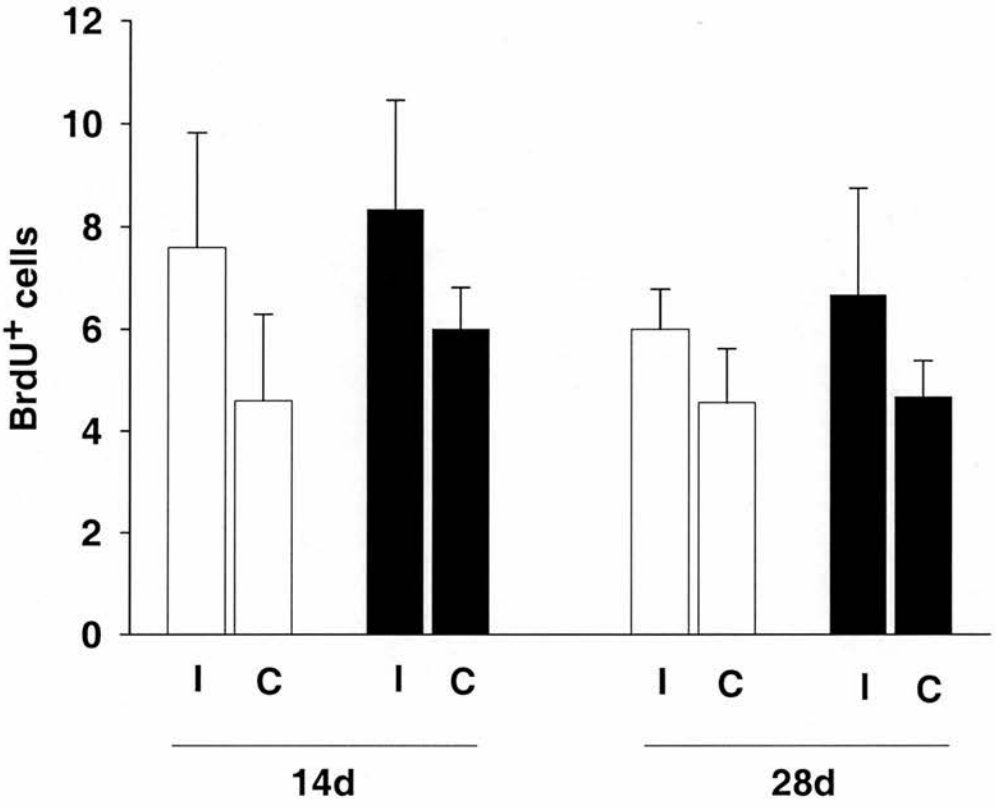


Figure 3.2.10 Neurogenesis in the dentate gyrus following chronic administration of LY404187

Data presented as mean ±SEM number of BrdU$^+$ cells in the sub-granular layer of the dentate gyrus (n=5-9,).. I=ipsilateral, C= Contralateral. Open bars = Vehicle, Closed bars = LY450108 (0.5mg/kg)

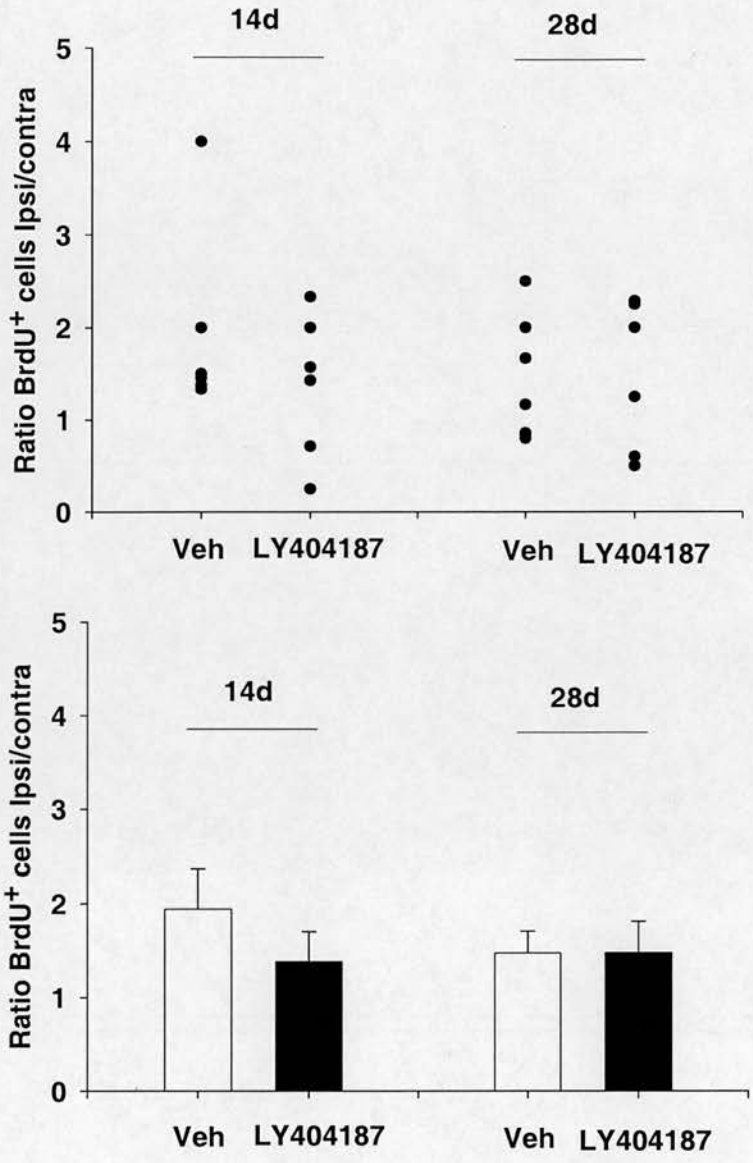


Figure 3.2.11 Ratio of Inter-hemispheric rates of neurogenesis

(Upper) Data presented as scatter graph of inter-hemispheric ratios in the rate of neurogenesis. (Lower) Data presented as mean ±SEM of inter-hemispheric ratios in the rate of neurogenesis (n=5-9,).

3.3 Biochemical changes following AMPA potentiation *in vivo*

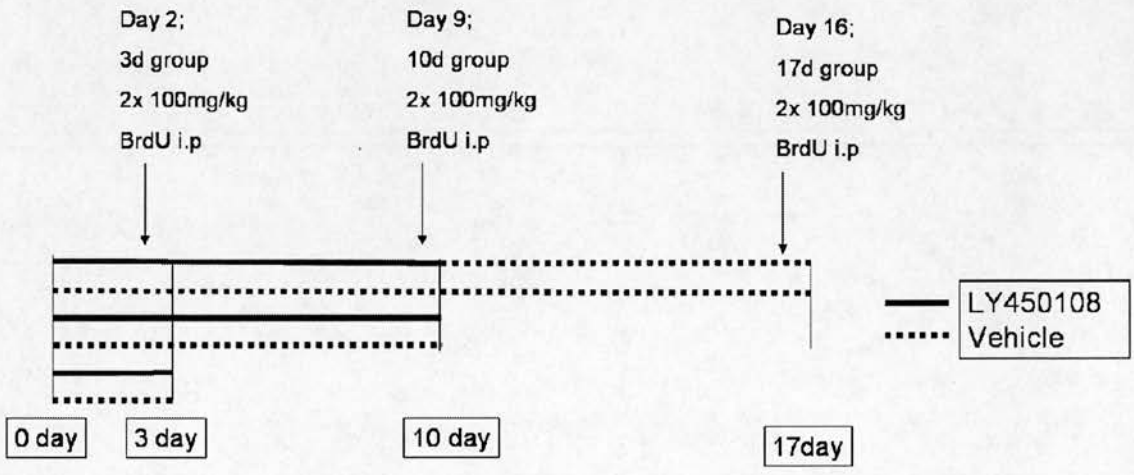
3.3.1 Aims

The aim of the three studies presented in this section was to establish whether chronic administration of an AMPA receptor potentiator, LY450108, could induce persistent changes in three key areas related to the actions of AMPA potentiators *in vivo*.

First, AMPA potentiators are thought to mediate their intracellular actions through the MAPK and CREB pathways. The level of phosphorylation of these proteins was measured by immunohistochemistry and western blotting in the hippocampus. Second, AMPA potentiators have been shown to be effective in classical animal models of depression. They also have been shown to promote sprouting of synapses positive for tyrosine hydroxylase, an enzyme required in the synthesis of dopamine. The levels of noradrenaline, serotonin and dopamine and their principal metabolites were measured by HPLC in the caudate nucleus, hippocampus and neo-cortex to determine whether long term LY450108 administration could change their levels. Third, AMPA potentiators have been shown to induce neurogenesis following chronic administration. The correlation between neurogenesis in the expression mood disorders and the actions of classical antidepressants has been well documented (Malberg, Eisch et al. 2000; Santarelli, Saxe et al. 2003).

Dosing protocol

The dosing protocols used in these studies were designed to allow the study of acute, chronic and persistent changes following the administration of LY450108. Animals were dosed twice daily for 3 days, 10 days and a final group had 10 day LY450108 administration and a further 7 days vehicle doses. Each group had compound and vehicle treatment groups of 6 animals each. Additionally, animals received doses of BrdU i.p. prior to being culled. In the neurogenesis study (shown below), 24 prior to being culled the animals were administered with 2 x 100mg/kg injections of BrdU i.p. In the neuronal development study animals are given 2 x100mg/kg injections of BrdU i.p 7 days before being culled (Except animals in the 3 days group, which receive injections on day 0, 30mins following the first injection of compound.



3.3.2 The effects of LY450108 on phosphorylation levels of ERK 1/2 and CREB in the hippocampus

AMPA potentiation increases the opening time of AMPA receptors. This can induce the direct induction of MAPKs and CREB through Lyn kinase and Ca^{2+} (through calcium permeable AMPA receptors) and indirectly through the recruitment of NMDA receptors. To investigate whether these pathways are induced *in vivo* I performed Western blots and immuno-histochemical analysis of levels of the phosphorylated proteins.

pERK 1/2

Levels of pERK 1/2 were assessed by measuring the intensity of pERK 1/2 chemiluminescence as a ratio of native ERK1/2 levels. Western blots identify two bands at 44 and 42 kDa. Equal loading was confirmed by the relative intensity of actin blots across wells. Levels of native ERK 1/2 were unchanged across all treatment groups. 6 animals per group were used, equal loading was assessed by protein assay and all measurements were blinded to treatment groups. Figure 3.3.1 shows a representative blot for pERK1/2 and ERK 1/2 and average ratio in intensities. Statistical analysis detected no significant change between treatment groups and time points (ANOVA $p>0.05$).

Immunohistochemistry allowed a more quantative measure of pERK levels and allowed localization of the positive cells (Figures 3.3.3-3.3.5)

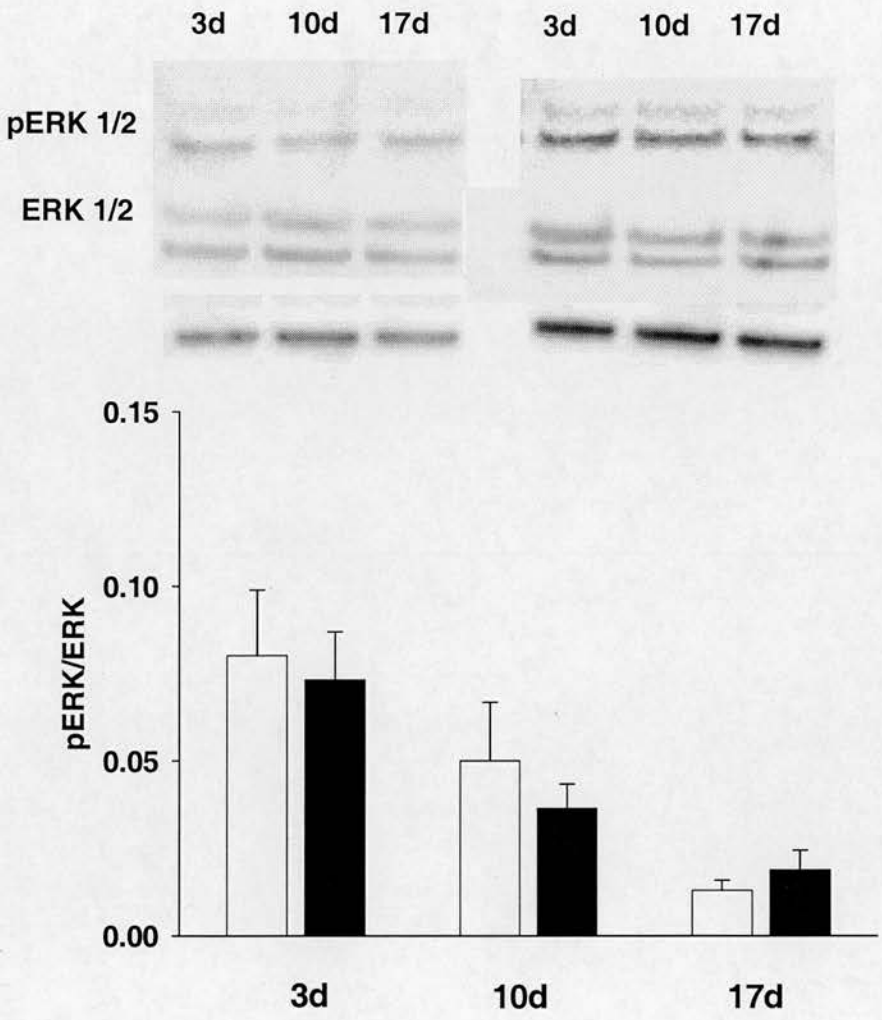


Figure 3.3.1 Levels of pERK 1/2 in the whole hippocampus following chronic administration of LY450108

(Upper) Representative Erk 1/2 western blots from vehicle (left) and LY450108 (right) showing pErk, native Erk and actin loading control (Lower) Data expressed as mean ±SEM fold change chemiluminescence levels of pERK 1/2 expressed as a ratio of chemiluminescence levels of native ERK 1/2. Levels of native ERK1/2 were unchanged across all goups (n=6, p>0.05). Open bars = Vehicle, Closed bars = LY450108 (0.5mg/kg)

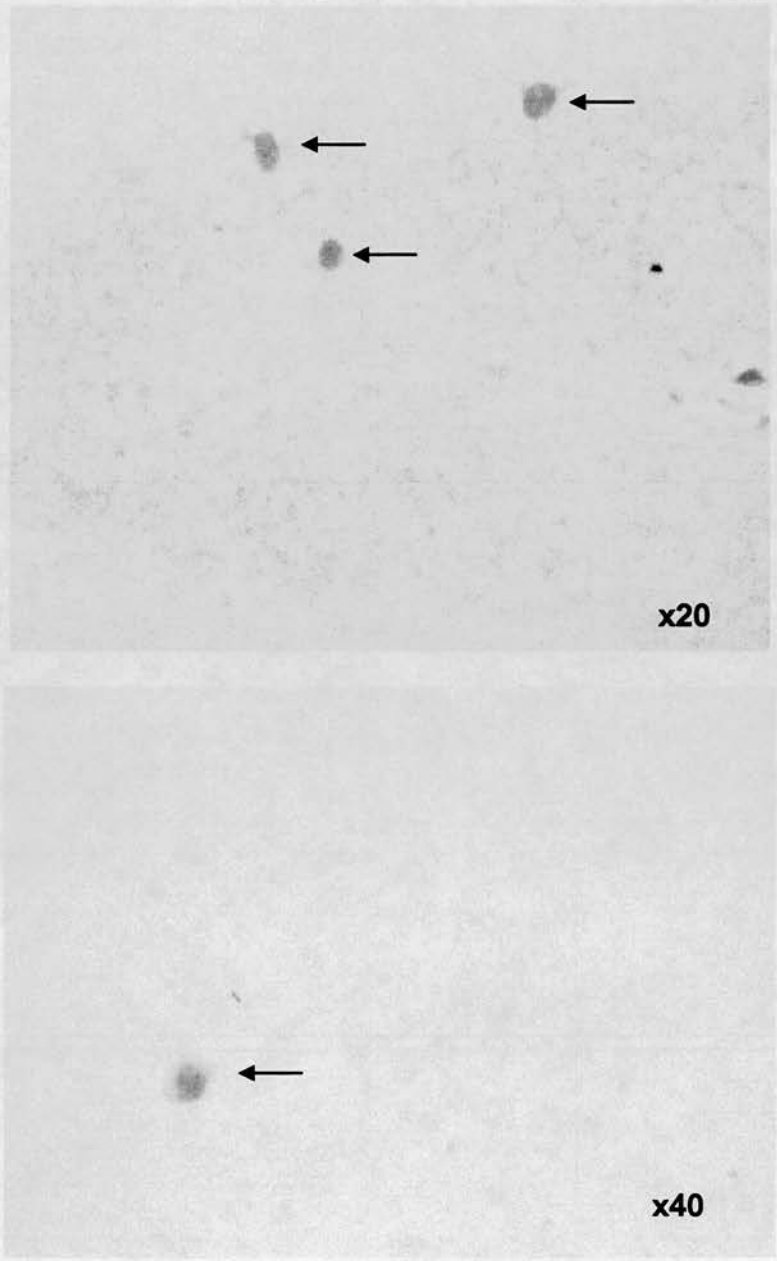


Figure 3.3.2 Detection of pErk 1/2^{+}ve cells in the hippocampus

(Upper) Low power micrograph showing the staining pattern of pErk^{+}ve cells (black arrows) in the dentate gyrus. (Lower) At a higher power positively stained cells (black arrows) could be clearly identified. Cells were counted blinded to treatment group.

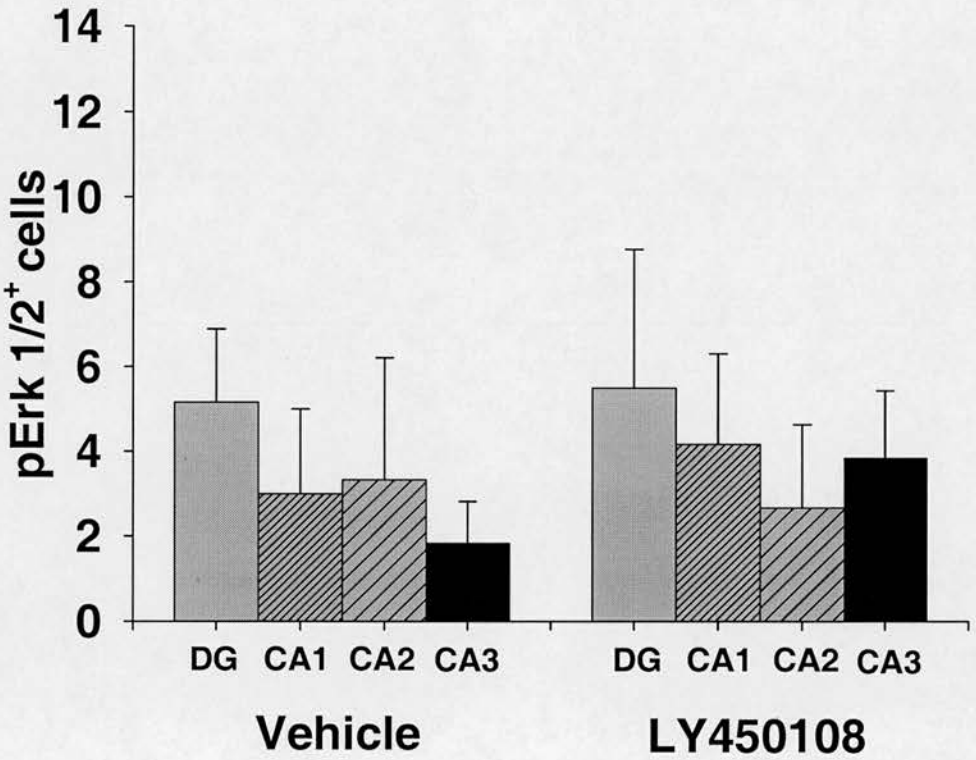


Figure 3.3.3 pERK 1/2 immunopositive cells in the hippocampus following 3 days administration of LY450108

Data presented as mean ±SEM (n=6, p>0.05). The number of pERK 1/2$^+$ cells in four regions of the hippocampus. There was no significant change in any region between vehicle and drug treated groups following 3 days chronic administration of LY450108 (0.5mg/kg).

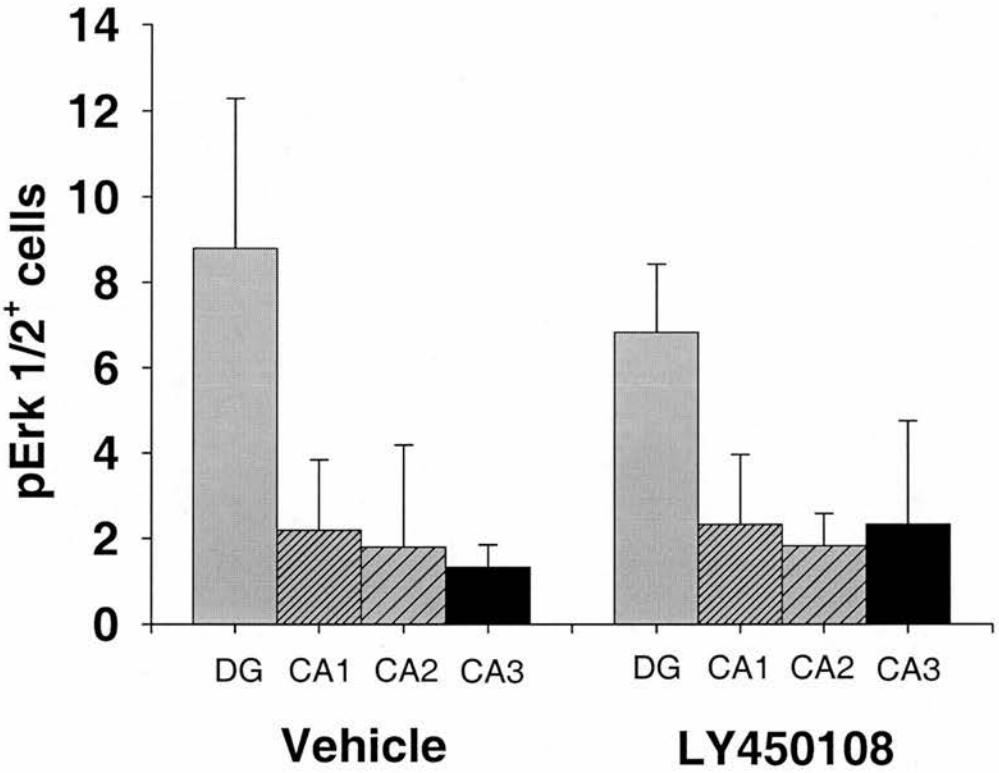


Figure 3.3.4 pERK 1/2 immunopositive cells in the hippocampus following 10 days administration of LY450108

Data presented as mean ±SEM (n=6, p>0.05). The number of pERK 1/2$^+$ cells in four regions of the hippocampus. There was no significant change in any region between vehicle and drug treated groups following 10 days chronic administration of LY450108 (0.5mg/kg).

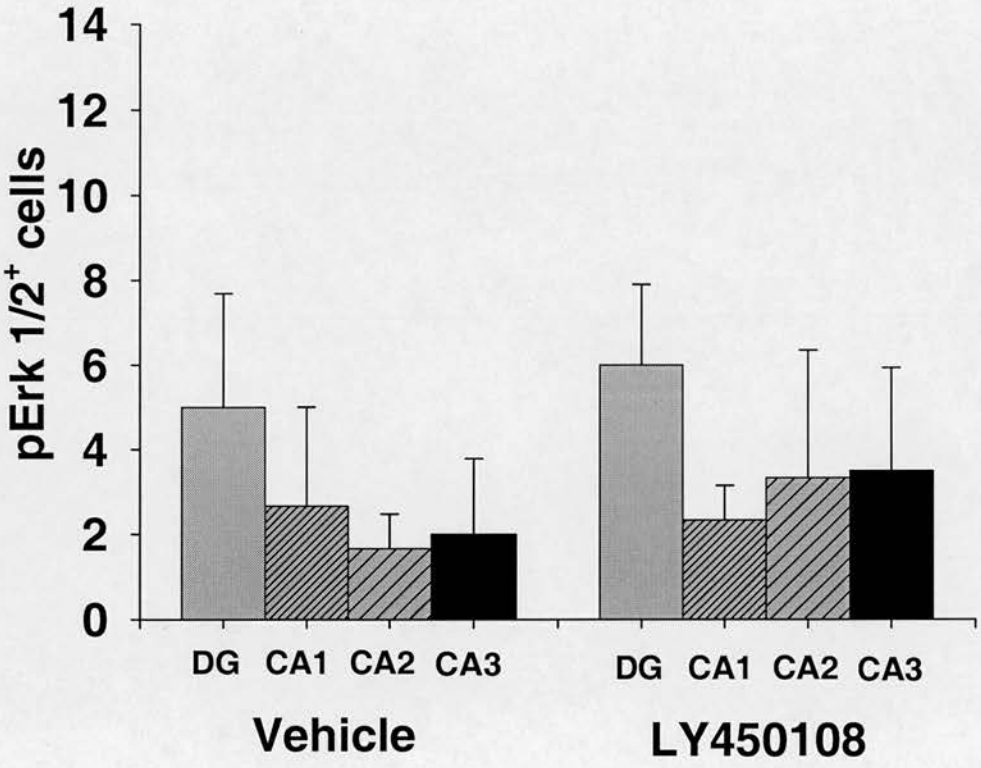
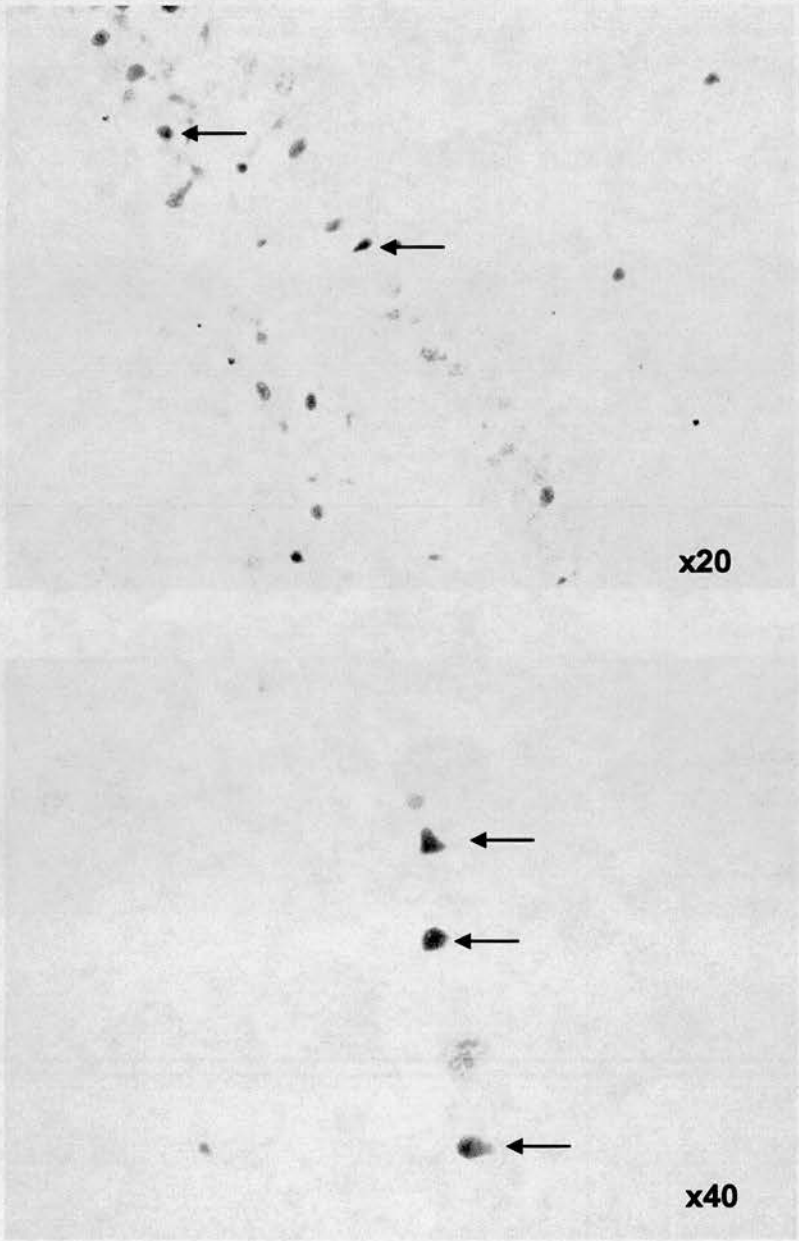


Figure 3.3.5 pERK 1/2 immunopositive cells in the hippocampus following 17 days administration of LY450108

Data presented as mean ±SEM (n=6, p>0.05). The number of pERK 1/2$^+$ cells in four regions of the hippocampus. There was no significant change in any region between vehicle and drug treated groups following 17 days chronic administration of LY450108 (0.5mg/kg).

pCREB

Measuring levels of pCREB by Western blotting was not possible due to the poor quality of the antibody in Western blotting. In contrast, the detection of $pCREB^{+}$ cells in paraffin embedded tissue by immunohistochemistry revealed clearly labelled cells along the dentate gyrus that were clearly quantifiable (Figure 3.3.6). Assessing the number of cells in the CA1, CA2 and CA3 regions however was not performed due the high background staining in this area, making the identification of positive cells impractical. The results show no significant change in the number of pCREB cells across all groups (Figure 3.3.7) (ANOVA, $p>0.05$).



3.3.6 Detection of pCREB^{+}ve cells in the hippocampus

(Upper) Low power micrograph showing the staining pattern of pCREB^{+}ve cells (black arrows) in the dentate gyrus. (Lower) At a higher power intensely stained cells (black arrows) could be clearly identified. Cells were counted blinded to treatment group.

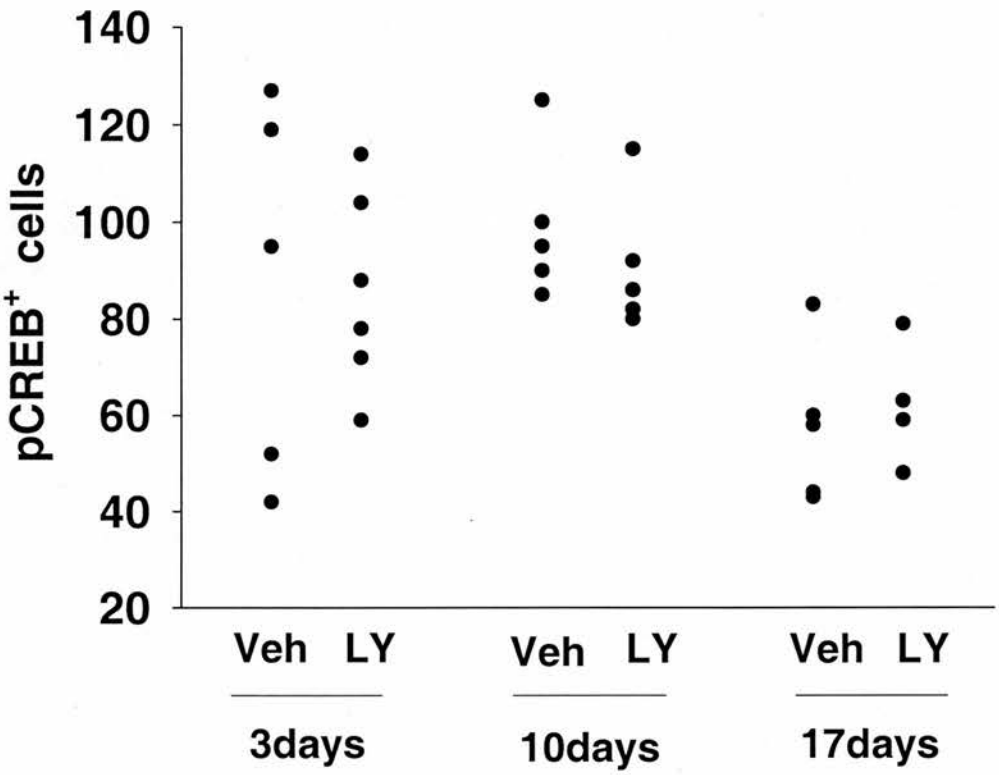


Figure 3.3.7 The number of pCREB$^+$ cells in the dentate gyrus following chronic administration of LY450108

Data are presented as a scatter graph of the number of pCREB$^+$ cells in individual mice (n=4-6, p>0.05) There was no change between vehicle and drug treated groups following 3, 10 or 17 days of drug administration. veh= vehicle, LY=LY450108 (0.5mg/kg)

3.3.3 The effect of LY450108 on monoamine levels in the hippocampus, caudate nucleus and neocortex

All antidepressants in widespread clinical use are able to enhance central levels of aminergic neurotransmitters. In this study the levels of 5-HT, NA and DA and their principal metabolites were measured across three brain regions by HPLC.

High levels of DA, DOPAC and HVA were detected in striatal tissue. Levels of 5HT, NA and 5HIAA were negligible (data not shown). There was no statistical difference between each of the detected compounds across treatment groups and time points (Figure 3.3.8) ($p>0.05$).

Low levels of 5HT, 5HIAA and NA were detected in the hippocampus, reflecting the prevalence of glutamatergic synapses in this structure. Negligible levels of DA and HVA were detected (data not shown). There were no significant changes between treatment groups and time points (Figure 3.3.9) $p>0.05$).

Approximately 10 times higher levels of 5HT and 5HIAA and NA were detected in the cortex than in the hippocampus. There where no significant changes between drug treatment groups at any time point ($p>0.05$). Due to technical problems the 3 and 10 day animals were not run contemporaneously with the 17 day group of animals (Figure 3.3.10).

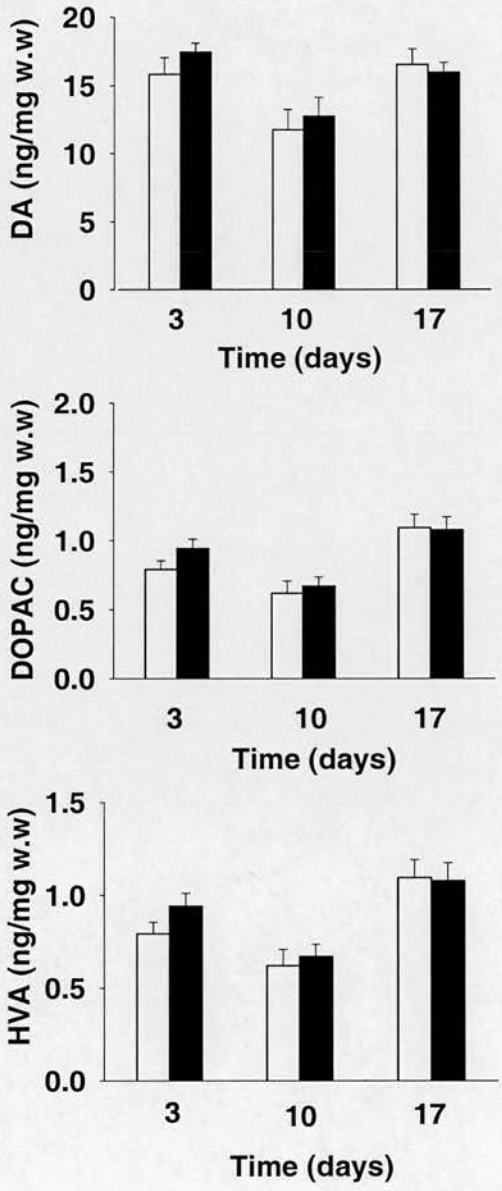


Figure 3.3.8 Monoamine levels in the caudate nucleus following administration of LY450108

Data are presented as mean monoamine levels ±SEM (n=6, p>0.05). There were no statistically significant differences between groups. Open bars= Vehicle, Closed Bars= LY450108 (0.5mg/kg)

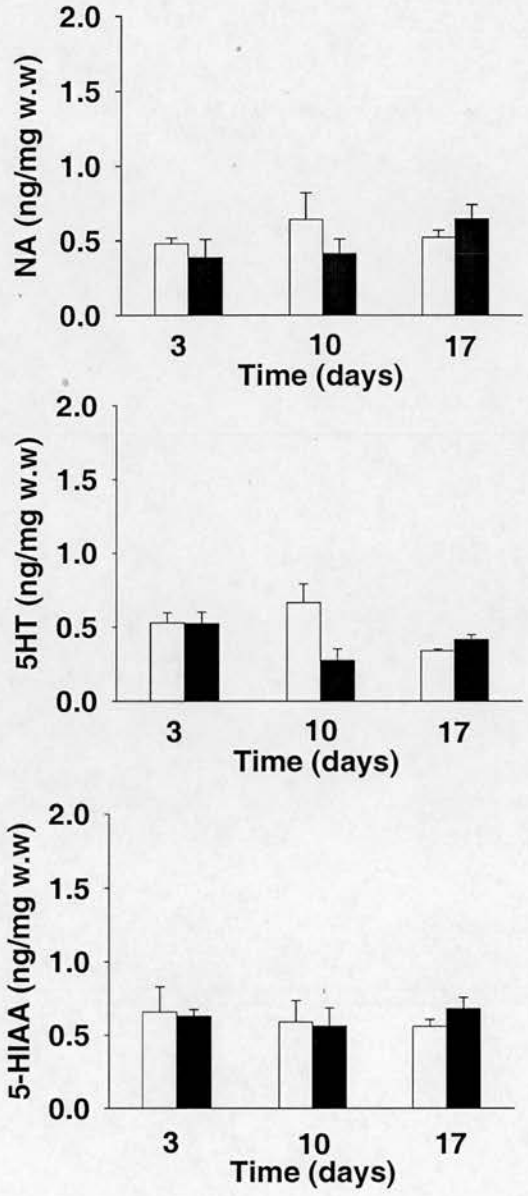


Figure 3.3.9 Monoamine levels in the hippocampus following administration of LY450108

Data are presented as mean monoamine levels ±SEM (n=6, p>0.05). There were no statistically significant differences between groups. Open bars= Vehicle, Closed Bars= LY450108 (0.5mg/kg)

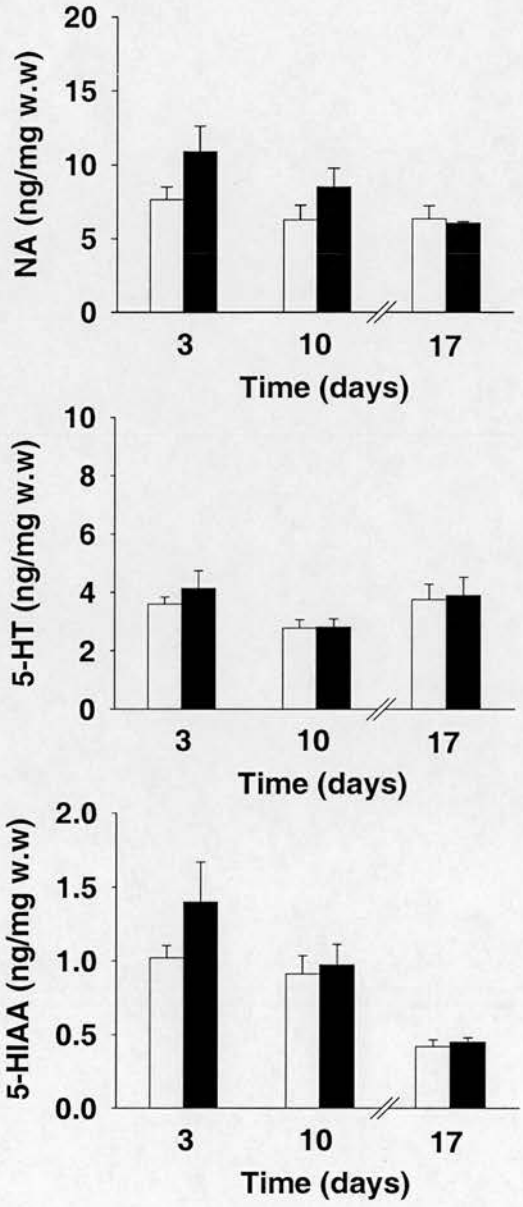


Figure 3.3.10 Monoamine levels in the neocortex following administration of LY450108

Data are presented as mean monoamine levels ±SEM (n=6-12, p>0.05). Open bars= Vehicle, Closed Bars= LY450108 (0.5mg/kg)

3.3.4 The effect of LY450108 on the rate of neurogenesis and neuronal development in the hippocampus

The final end point of this study was the assessment of the rate of neurogenesis and neuronal development in the hippocampus following LY450108 administration. Animals were dosed with BrdU 24hrs, 3 days of 7 days prior to being culled (see materials and methods). The rate of neurogenesis was assessed by counting the number of $BrdU^+$ cells present in the subgranular zone of the dentate gyrus. The average number of cells per slide was unchanged across treatment groups and time points. There is a non-significant increase ($p=0.08$) in LY450108 treated 17 day group (veh=9±1.6 LY450108=16±2.9) (Figure 3.3.11).

The differentiation of stem cells into neuronal cells was assessed using the neuronal marker NeuN. $BrdU^+/NeuN^+$ positive cells could be identified at high power as the diffuse, cytoplasmic staining of NeuN could easily be distinguished from the nuclear staining of BrdU (Figure 3.3.12). The end point was the percentage of $BrdU^+$ cells that were also $NeuN^+$. Statistical analysis revealed no change between treatment groups and time points (Figure 3.3.13).

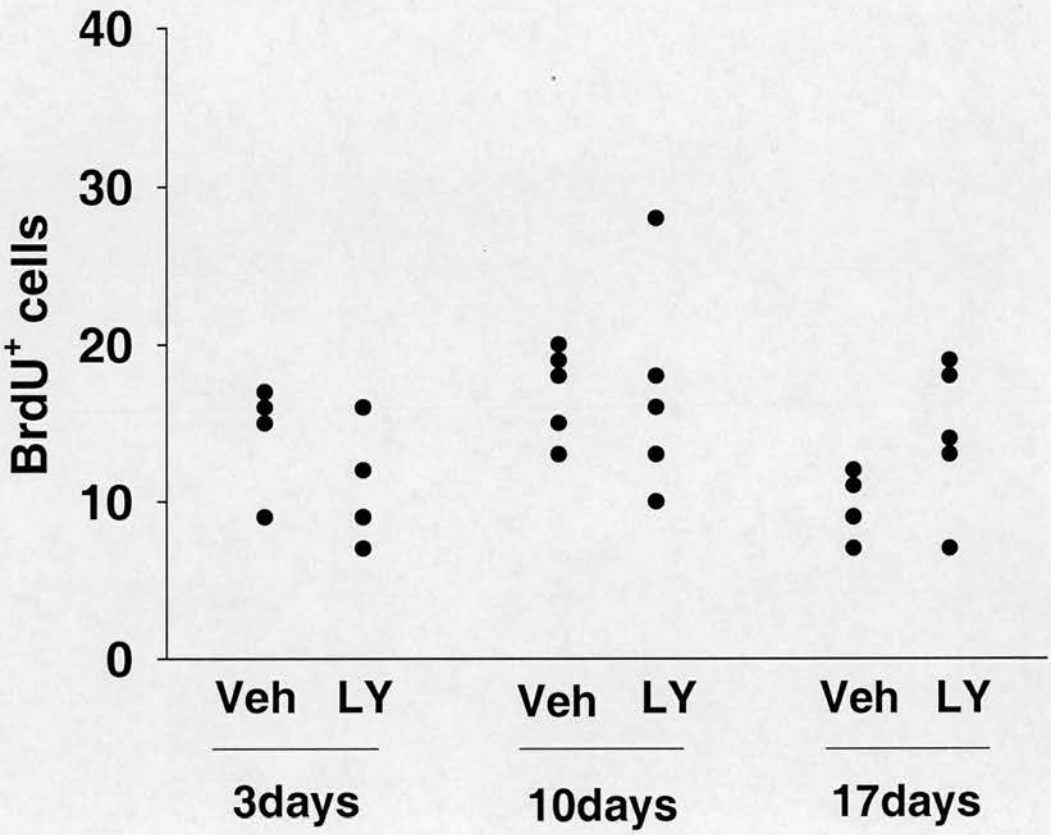


Figure 3.3.11 Neurogenesis following chronic administration of LY450108

Data are presented as scatter graph of raw data of $BrdU^+$ cells (n=4-6, p>0.05) For each animal the average of two coronal sections through the hippocampus was measured. There was no change between vehicle and drug treated groups following 3, 10 or 17 days of drug administration. veh= vehicle, LY=LY450108 (0.5mg/kg).

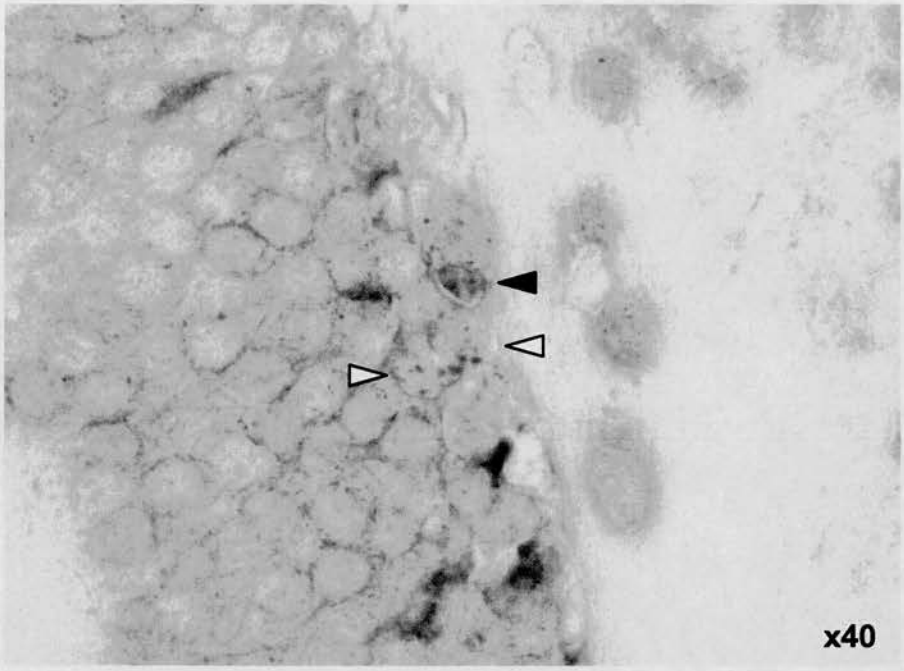


Figure 3.3.12 Detection of neuronal development in the hippocampus

At high magnification cells stained positive for BrdU (brown) and NeuN (blue) can easily be identified. Cells displayed intensely stained cell nucleus surrounded by blue cytoplasm (red arrows), or a punctuate staining of the nucleus surrounded by blue cytoplasm (white arrows)

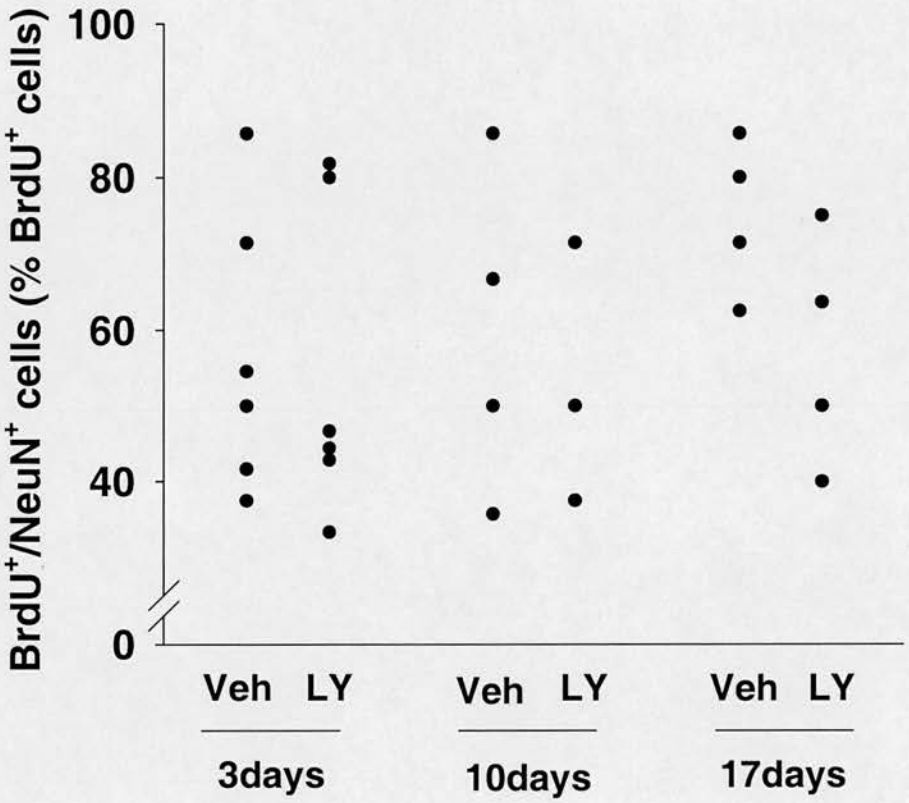


Figure 3.3.13 Neuronal development following chronic administration of LY450108

Data are presented as scatter graph of raw data of $BrdU^+$ cells (n=4-6, p>0.05). For each animal the average of two coronal sections through the hippocampus was measured. There was no change between vehicle and drug treated groups following 3, 10 or 17 days of drug administration. veh= vehicle, LY=LY450108 (0.5mg/kg).

4. Discussion

4.1 Neurite growth with LY404187

4.1.1 Summary

The data presented in Chapter 3.1 show that the AMPA receptor potentiator LY404187 can increase the average length of neurites and the expression of the cytoskeletal protein neurofilament in SH-SY5Y cells. Further experiments suggested that the increase in neurite length was mediated in part by the neurotrophin BDNF.

4.1.2 Methodology

The primary endpoint of the studies presented in Chapter 3.1 was the change in average neurite length in SH-SY5Y cells. The choice of SH-SY5Y cells as the model system for these experiments was made on the basis of their human origin, their established use in neurite growth studies and their availability. Pilot experiments were also carried out in cerebellar granule cells and hippocampal neurons with the aim of establishing a neuronal model in which to study the molecular mechanisms of AMPA potentiation and neurite growth. The cerebellar granule cells were not pursued further, as the density required to promote adequate cell survival and neurite growth did not permit accurate measuring of neurite growth (data not shown). Dissociated hippocampal neurons collected from E16-18 rats provided healthy, dispersed and neurite bearing cells ideal for exploring the mechanisms of AMPA receptor mediated intracellular cascades. However following several pilot experiments aimed at investigating down stream effects of AMPA potentiation they were not pursued due to extreme sensitivity to excitotoxicity.

4.1.3 Discussion

AMPA receptor potentiators were conceived as compounds that could facilitate the induction of LTP and were hypothesised to act as cognitive enhancers by promoting memory formation (Granger, Staubli et al. 1993; Staubli, Rogers et al. 1994; Lynch 2004). Many compounds, termed AMPA potentiators, of various chemical classes have indeed been shown to act as cognitive enhancers in a variety of animal and human paradigms (Larson, Lieu et al. 1995; Granger, Deadwyler et al. 1996; Lynch, Granger et al. 1997; Hampson, Rogers et al. 1998; Buccafusco, Weiser et al. 2004; Porrino, Daunais et al. 2005). In these studies the effects of tested compounds have been short lived, leaving no residual effect following withdrawal of compound, implying that cognitive enhancement by AMPA potentiation may only have symptomatic improvement for cognitively impaired individuals. However, more recent studies suggest that AMPA potentiation may also have neurotrophic and neuroprotective properties (Dicou, Rangon et al. 2003; O'Neill, Murray et al. 2004; O'Neill, Murray et al. 2005). In particular studies carried out by O'Neill and colleagues suggest enhanced sprouting of dopaminergic terminals in the rat striatum following 6-OHDA lesions.

The studies described in chapter 3.1 were aimed at investigating the mechanisms of any structural changes induced by AMPA potentiation. Our findings demonstrate that direct application of an AMPA receptor potentiator can induce BDNF-dependent morphological plasticity.

A role of glutamate receptors in regulating neurite growth is well established but varied. Glutamate application inhibits neurite growth in embryonic rat motor neurons

through Ca^{2+} permeable AMPA receptors (Metzger, Wiese et al. 1998), whilst kainic acid promotes neurite growth in DRG cells in the presence of corticosterone (Tsai, Chiu et al. 2002) and AMPA receptor activity stabilises actin dynamics in growth cones of developing axons (Chang and De Camilli 2001). The only evidence of an AMPA potentiator inducing neurite growth, published over a decade ago, reported moderate enhancement of neurite length in murine CGNs with the weak AMPA potentiator aniracetam (Fushiki, Matsumoto et al. 1995). However a number of recent reviews have supported the possibility that AMPA potentiation may be able to enhance structural plasticity in the adult CNS (O'Neill, Bleakman et al. 2004; Alt, Nisenbaum et al. 2006).

To explore the effects of AMPA potentiation on structural plasticity we developed an assay for the measurement of neuritic growth *in vitro* using the human SH-SY5Y neuroblastoma cell line. SH-SY5Y cells possess biochemical characteristics similar to dopaminergic cells, such as tyrosine-β-hydroxylase activity (Oyarce and Fleming 1991) and are a common model for the investigation of neuronal differentiation and neurite growth (Ivankovic-Dikic, Gronroos et al. 2000; Cui, Yang et al. 2003; Price, Yamaji et al. 2003; Jamsa, Hasslund et al. 2004; Daniel, Mudge et al. 2005). They express AMPA receptor subunit mRNA, Trk-B receptors and BDNF protein (Christnacher and Sommer 1995; Ho, Eggert et al. 2002; Olivieri, Otten et al. 2003; Ruiz-Leon and Pascual 2003).

Our results demonstrate that LY404187 in the presence of s-AMPA induced neurite outgrowth in SH-SY5Y cells. The magnitude of the effect was comparable to that induced by BDNF incubation and around half that induced by NGF and retinoic acid. LY404187 thus compares favourably to classical neurite growth inducers,

especially when considering that an allosteric modulator of an ion channel is being compared to neurotrophins and a powerful differentiation factor. This growth was dependent on AMPA receptors, as incubation with CNQX, a competitive AMPA receptor antagonist attenuated the response.

In addition to monitoring neurite growth by morphometric analysis we also investigated changes in neurofilament expression. The cytoskeletal protein neurofilament has been widely used as an indicator of neurite growth and maturation (Drejer and Honore 1988). We observed increases in expression following incubation with LY404187 alone and co-incubation with s-AMPA and LY404187. In all experiments the largest increase was seen with co-incubation of LY404187 and s-AMPA. LY404187 has no intrinsic activity at AMPA receptors when assessed by electrophysiological recordings of transfected cell lines and neuronal cells (Gates, Ogden et al. 2001; Miu, Jarvie et al. 2001). However long term administration in cell culture systems by other biarylproposulphonamides is able to elicit intracellular responses when applied on their own. This is probably due to their ability to potentiate AMPA receptors in the presence of low levels of glutamate present in the medium, released by cells, in the case of glutamatergic neurons, or released into the medium following cell death and lysis (Legutko, Li et al. 2001). Increased neurofilament expression following incubation with LY404187, where no effect was seen on neurite outgrowth, may therefore reflect the higher sensitivity of Western blot analysis (Doherty, Dickson et al. 1984; Cambray-Deakin, Morgan et al. 1987; Encinas, Iglesias et al. 2000). Taken together the NF-H expression and the neurite outgrowth data strongly suggest that AMPA potentiation can promote structural changes *in vitro*.

The link between synaptic activity and neurotrophin expression is well established (Zafra, Hengerer et al. 1990; Ghosh, Carnahan et al. 1994; Tao, Finkbeiner et al. 1998; Limatola 2004). In particular AMPA receptor activity can depolarise membrane potentials recruiting VGCC and NMDA receptors which in turn increase intracellular levels of $[Ca^{2+}]_i$. Through the recruitment of a number of calcium sensitive enzymes, including CaM and CaMKs this can lead to the phosphorylation of CREB and the increased synthesis of BDNF (Carlezon, Duman et al. 2005) (Figure 3.11). As well as gating Ca^{2+} ion flux through GluR2 lacking receptors AMPA receptors can recruit a variety of second messenger molecules such as the non-receptor tyrosine kinase Lyn (Hayashi, Umemori et al. 1999), FAK (Millan, Aguilar et al. 2001), PI(3)K (Millan, Arias-Montano et al. 2004) and G-proteins (Wang, Small et al. 1997). Through these they can recruit intracellular signalling cascades such as the MAP kinase pathways (Wang and Durkin 1995; Hayashi, Umemori et al. 1999; Perkinton, Sihra et al. 1999; Bahr, Bendiske et al. 2002) and enhance gene expression through the activation of transcription factors such as c-fos and CREB (Sassone-Corsi, Visvader et al. 1988; Sheng, McFadden et al. 1990; Rajadhyaksha, Barczak et al. 1999; Garcia, Anderson et al. 2003; Fowler, Whalley et al. 2004).

CREB is expressed in all cells of the brain and is regulated by many complex region, cell and signal specific pathways and is a key link in the chain of membrane to nucleus signal transduction, crucial for sensing changes in the extracellular environment and translating them into changes in gene transcription (Carlezon, Duman et al. 2005). Phosphorylation of CREB can regulate the expression of a large set of genes the most studied of which include BDNF, Tyrosine hydroxylase, Bcl-2, GluR1 and c-fos (Zafra,

Hengerer et al. 1990; Finkbeiner, Tavazoie et al. 1997; Shieh, Hu et al. 1998; Tao, Finkbeiner et al. 1998). Due to its distribution and range of target genes CREB activity has been implicated in mediating a number of processes related to memory, cognition, the pathology and treatment of depression, addiction and anxiety (Nestler, Barrot et al. 2002; Scott, Bourtchuladze et al. 2002; Carlezon, Duman et al. 2005).

BDNF signals primarily through the tyrosine kinase receptor Trk-B (Glass, Nye et al. 1991; Klein, Nanduri et al. 1991; Soppet, Escandon et al. 1991; Squinto, Stitt et al. 1991; Barbacid 1995). Western analysis for Trk receptor expression revealed that LY404187 in the presence of AMPA can increase expression levels, suggesting an increase in neurotrophin responsiveness. There is evidence for the production of BDNF by SH-SY5Y cells under basal conditions (Ruiz-Leon and Pascual 2003) and my results strongly indicate that neurite growth associated with AMPA potentiation is dependent on BDNF. BDNF has been identified over the past decade as a modulator of a number of cellular processes including early CNS development (Legutko, Li et al. 2001), synaptic transmission (Ernfors, Lee et al. 1994) and regeneration following brain injury (Patterson, Abel et al. 1996; Schratt, Nigh et al. 2004). It has also been hypothesised that increases in growth factors such as BDNF may lead to the beneficial effects of AMPA potentiation in a range of animal models (Ebadi, Bashir et al. 1997). In the present study I demonstrated that BDNF antibody-sequestration attenuates the increase in neurite length following co-incubation with AMPA and LY404187 indicating that BDNF is a potential downstream effector of neurite growth following AMPA receptor potentiation. AMPA receptor potentiation has recently been shown to protect cells from excitotoxic cell death via Lyn, ERK1/2, CREB and BDNF activity (Wu, Zhu et al.

2004). A similar pathway is likely to be activated in our model to promote neurite outgrowth (O'Neill, Bleakman et al. 2004).

Enhancing central plasticity, neuritic sprouting and regeneration are promising therapeutic strategies for the treatment of a number of neurological conditions including depression and Parkinson's disease (Manji, Quiroz et al. 2003; Fernandez-Espejo 2004; Mueller, Mack et al. 2005). Reductions in volume and structural abnormalities in a number of brain regions have been observed in humans with depression (Sheline 2003) and reduced neurogenesis (D'Sa and Duman 2002), decreased levels of BDNF (Angelucci, Brene et al. 2005) and plasticity imbalances (Peled 2005) have also been implicated. Neuroprotective and neurotrophic strategies are being investigated for the treatment for Parkinson's disease, focusing on the actions of the neurotrophins BDNF and GDNF (Mamounas, Blue et al. 1995; Gash, Zhang et al. 1998; Fernandez-Espejo 2004). Biarylproposulphonamides have been demonstrated to enhance neurogenesis (Bai, Bergeron et al. 2003), BDNF expression *in vivo* (Mackowiak, O'Neill et al. 2002), sprouting of nigro-striatal dopamingergic terminals and show behavioural effects in animal models of depression (Li, Tizzano et al. 2001) and Parkinson's disease (O'Neill, Murray et al. 2004; O'Neill, Murray et al. 2005). Along with the present demonstration that they can promote structural plasticity, biarylproposulphonamides show a favourable profile for the treatment of a number of neurological conditions.

4.1.4 Conclusions

Our results further highlight the long term neurotrophic effect of AMPA receptor potentiation and support evidence that, in addition to transient cognitive enhancement (O'Neill, Bleakman et al. 2004), AMPA receptor potentiators can promote downstream plasticity, via the actions of neurotrophins, that may be beneficial for the treatment of chronic neurodegenerative disorders.

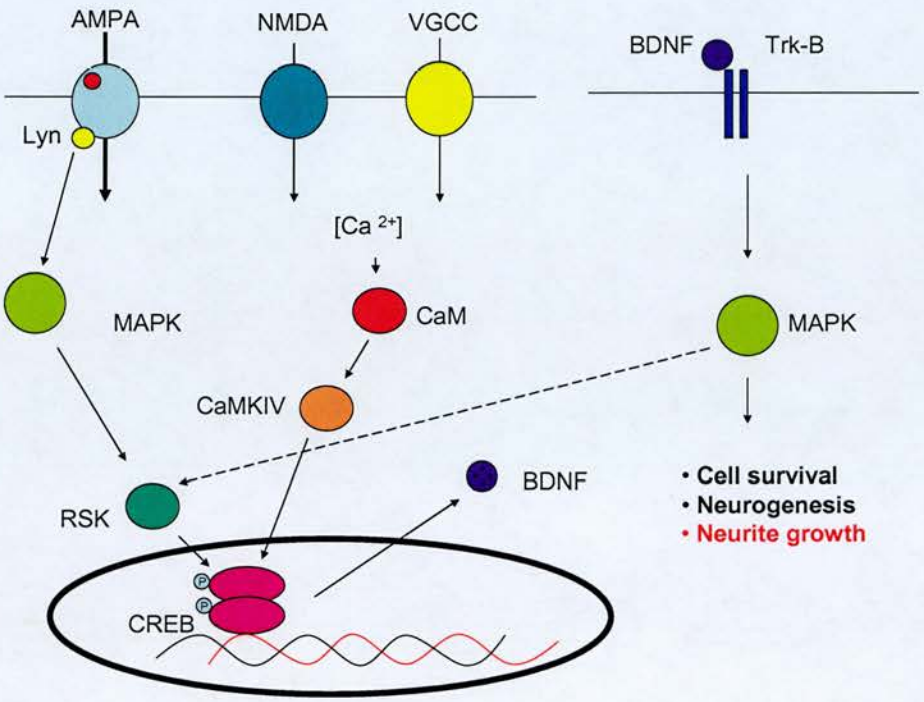


Figure 4.1.1 *Working hypothesis for the mechanisms of action of AMPA potentiators. Enhanced periods of AMPA receptor activation promote the recruitment of NMDA and VGCC through increased membrane depolarisation. Elevated levels of Ca^{2+} activate the CaM-CaMK cascade which results in phosphorylation of CREB. AMPA receptors can also directly recruit MAPK pathways and CREB activation through non receptor tyrosine kinase Lyn. CREB regulates the expression of a number of genes including BDNF which is secreted and can act in an autocrine or paracrine fashion, through Trk-B receptors to promote a number of neurotrophic responses including the neurite growth described in this chapter.*

4.2 Neuronal sprouting in dentate gyrus following ECL

4.2.1 Summary

The data from this study indicate that 14 and 28 days of twice daily injections of LY404187 (0.5mg/kg) had no effect on lesion volume or the ratio of synaptophysin immunoreactivity between hemispheres in the molecular layer of the dentate gyrus following ECL. Thus, my hypothesis that LY404187 would enhance the recovery of synaptophysin immunoreactivity following lesioning was not supported in this study. There are three major reasons for which this might be the case: the experiment was flawed, through practical or statistical reasons; the dosing protocol of LY404187 chosen was not able to induce the appropriate changes in brain levels of LY404187 or AMPA potentiation may not be able, as hypothesised, to induce significant sprouting and synapse formation in this paradigm.

4.2.2 Methodology

Unilateral ibotenic acid injections in the entorhinal cortex were successful in producing excitotoxic lesions in the ventro-caudal portion of the entorhinal cortex. Histological analysis revealed staining showing that the tissue was deeply necrotic and contained many shrunken intensely stained cell bodies and multiple holes in the tissue representing widespread and substantial tissue damage. 3D reconstruction of the lesion site for the LY404187 study and quantification of the volume showed that the procedure was highly reproducible both in terms of the location of the lesion and its volume. This type of lesion leads to a loss of synapses in the ipsilateral middle molecular layer of the

dentate gyrus. The pilot study revealed a significant difference in the ratio of intrahemispheric synaptophysin O.D. between 3 and 7 days post lesion. Animals in the 28 days recovery group did not show significantly altered levels of synaptophysin. The lesions themselves produced small changes in synaptophysin levels and there were no changes in the overall O.D levels between hemispheres. This is in direct contrast to published data that showed maximum loss in synaptophysin occurs 28 days following ECL (White, Nicoll et al. 2001). Histological analysis of the entorhinal cortex from animals in the study showed substantially smaller lesions in the 28 day group than in other groups (data not shown). Animals from each group of all underwent surgery on the same day could and the small lesions in the 28 days group could have been due to a faulty batch of ibotenic acid used that day. In, the follow-up study which examined the effects of LY404187 in the lesioned mice, mice from the same recovery groups were all lesioned on to ensure that animals from any one group were not all lesioned on the same day.

4.2.3 Discussion

LY404187 and other biarylproposulphonamides of its class readily diffuse across the blood brain barrier and have been shown, at a concentration of (0.5mg/kg), to elevate cerebral glucose utilisation in the brain of the rat (Fowler, Whalley et al. 2004). At this dose LY404187 has also induced positive responses in a number of behavioural models and was the optimum dose for promoting functional and histological recovery in a 6-OHDA model of Parkinson's Disease (Quirk and Nisenbaum 2002; O'Neill, Murray et al. 2004). Although the exact pharmacokinetics of LY404187 in mice are not known,

particularly those associated with chronic administration, given the available literature the dose of 0.5mg/kg was judged to be sufficient to enhance central levels of BDNF.

Given that the surgery was a practical success and induced a substantial loss of synaptophysin in the dentate gyrus and that the dosing regime was the appropriate one based on the available literature on LY404187, we can conclude that AMPA potentiation had no appreciable effect in the model for valid physiological reasons.

Firstly we can conclude that AMPA potentiation does not exacerbate the damage caused by ibotenic acid lesions. One of the main areas of concern with AMPA potentiators is the potential to induce excitotoxicity, indeed IDRA-21 had been shown to exacerbate excitotoxic hippocampal injury *in vivo* (Yamada, Covey et al. 1998), although this was probably due to non specific kainate receptor effect. However it seems that AMPA potentiation can actually protect against excitotoxicity both *in vitro* (Wu, Zhu et al. 2004) and ischemic damage *in vivo* (Dicou, Rangon et al. 2003).

Levels of synaptophysin immunoreactivity in the dentate gyrus show no change across all groups indicating no significant effect on the replacement of lost synapses in this area. The hypothesis for this study expanded on three principal observations from the literature and from my own work. Experiments in rats using 6-OHDA lesion of the nigro-striatal tract show enhanced TH immunoreactivity in the striatum following AMPA potentiator administration. The authors suggest that this is due to the sprouting of surviving dopaminergic terminals. The study also showed that AMPA potentiation could act in a neurotrophic as well as neuroprotective manner, as effects were seen even if treatment was delayed up to 16 days post surgery (O'Neill, Murray et al. 2004; O'Neill, Murray et al. 2005). AMPA potentiators of a range of classes and potencies

have been shown to enhance the expression of BDNF *in vivo* and *in vitro*, particularly in the dentate gyrus (Legutko, Li et al. 2001; Mackowiak, O'Neill et al. 2002; Lauterborn, Truong et al. 2003). Observations made from my work *in vitro* show that AMPA potentiation can induce cytoskeletal change that is dependant on BDNF (Voss, Milne et al. 2006). BDNF has wide ranging developmental, neurotrophic and homeostatic properties and could be expected to enhance structural recovery following injury and synaptic loss (Cohen-Cory and Fraser 1995; Binder 2004; Binder and Scharfman 2004; Du and Poo 2004).

There are three major physiological reasons why the results of this experiment did not confirm my hypothesis. First is the time course of the experiment. The time points of 14 and 28 days were chosen because they represented the points at which there was likely to be the highest levels of synaptic loss in the hippocampus (White, Nicoll et al. 2001). At these time points it is reasonable to presume that any structural effect would be apparent. However if one considers the natural progression of the lesion (7-28 days of degeneration followed by a prolonged period of regenerative sprouting), it could be argued that trying to promote sprouting at such an early stage, when the synapses themselves are still degenerating is physiologically impossible. The up-regulation of BDNF might accelerate the sprouting process once begun, but might not be able, either because the levels are too low or because they are not specific both topographically and biologically to initiate the process. The issue of topography is a second important consideration. Studies *in vivo* have repeatedly shown that AMPA potentiation up-regulated BDNF expression predominantly in the dentate gyrus (Mackowiak, O'Neill et al. 2002; Lauterborn, Truong et al. 2003). Elevation of BDNF messenger and protein in

the cell bodies of the post synaptic cells may not be sufficient to promote sprouting and the formation of new synapses onto that cell. The processes that regulate the formation of new synapses are under intense investigation and it is still unclear what the role of the post synaptic cell is in this process. Evidence has suggested that retrograde signalling of BDNF has a homeostatic mechanism (Poo 2001). Therefore the enhanced levels of BDNF present and secreted by the granule cells may promote the formation and maintenance of synapses. However it is not clear whether AMPA potentiation enhances the expression of BDNF in other regions of the brain from which the sprouting occurs. These include the septum and commissural fibres. Assuming that BDNF does promote the growth and sprouting of neurites then the presence of AMPA receptors on cells in these areas could lead one to speculate that AMPA potentiation would indeed enhance BDNF and sprouting/growth in this area. The issue of the distribution of AMPA receptors in the regions of interest are an important issue in this study. Cells in the hippocampus express high levels of AMPA receptors and would therefore respond to AMPA potentiators, however what about the expression of AMPA receptors in other regions? AMPA receptors are widespread throughout the mouse brain but are concentrated in regions with high density of excitatory connections including the neo-cortex, hippocampal formation and dorso-lateral septum with lower levels in the striatum (Monaghan, Yao et al. 1984; Keinanen, Wisden et al. 1990). One would expect therefore that AMPA receptor potentiation in these areas would indeed enhance BDNF in some of the areas which participate in the restoration of the synaptic density in the dentate gyrus. However it should be noted that LY404187 has a 10 fold range on selectivity across GluR subtypes and its actions *in vivo* will be largely determined by

receptor stoichiometry. Finally it should be noted that the synaptophysin levels in the undamaged, contralateral, hemisphere were not measured. It is not clear whether AMPA potentiators are able to produce changes in undamaged tissue or are only able to promote recovery following degeneration. It is possible therefore that the effects of LY404187 in promoting the increase in ipsilateral synaptophysin levels were masked by contralateral increases too.

Neurogenesis in the adult brain occurs at a substantial rate in the sub-ventricular zone (SVZ) and the sub-granular layer of the dentate gyrus (SGL) (Gage 1998; Alvarez-Buylla and Garcia-Verdugo 2002). There have been reports of new neurons being generated in other brain regions but these reports remain largely unconfirmed and probably reflect very low physiological levels of neurogenesis (Gould, Reeves et al. 1999; Magavi, Leavitt et al. 2000). The functional significance of adult neurogenesis in the dentate gyrus is not fully understood (Kempermann, Wiskott et al. 2004), despite the identification of numerous external and internal signals that regulate the rate of cell proliferation and the specific cues that determine the development of newly generated neurons and the extent to which they integrate into local circuits. These include; environment enrichment (Kempermann, Gast et al. 2002), running (van Praag, Kempermann et al. 1999) and social stress (Gould, McEwen et al. 1997), epilepsy (Parent and Lowenstein 2002), certain brain traumas (Dash, Mach et al. 2001), steroids (Gould 1994; Karten, Olariu et al. 2005) growth factors (Pencea, Bingaman et al. 2001) and NMDA receptor activation (Cameron, McEwen et al. 1995). Glutamatergic input is therefore important in regulating neurogenesis. Lesioning the entorhinal cortex in rats and blocking NMDA receptors has been shown to increase the rate of neurogenesis

(Cameron, McEwen et al. 1995). Lesions of the granule cell layer also induced proliferation and the generation of new granule cell pre-cursors (Gould and Tanapat 1997). However electrical stimulation of the perforant path and hippocampal kindling and kainate induce seizures both increase neurogenesis (Gray and Sundstrom 1998). More recently studies have shown that lesions of both cortical and septal inputs into the brain transiently decrease the rate of neurogenesis. Our model was aimed at monitoring any permanent change in the rate of neurogenesis and whether this change was either reversed or enhanced by the administration of LY404187. The data in this thesis reveal that at the level of the lateral habenulae there is no change in the rate of neurogenesis across all groups, both in response to the lesion over time and following the administration of the AMPA potentiation. Most available data indicates that the effect of de-afferentation of the DG is transient and occurs in the first week following the lesion. If the change in proliferation rate is a response to the synaptic degeneration then at 14 and 28 days post lesion the degeneration of the synapses is complete and no effect would be evident.

AMPA potentiators in other models have been shown to induce cell proliferation following both acute and chronic dosing. The active isomer of LY404187, LY451646, was able to enhance cell proliferation up to 45% following 21 days of administration (Bai, Bergeron et al. 2003). The difference here could be due to species differences in availability of the compound. There are also differences in the method for measuring proliferation between the two studies. Our use of 50mg/kg is the most commonly used dose (Gould and Gross 2002). However there were only 4-9 $BrdU^+$ cells per section due to the thinness of the tissue. The experiment therefore was likely to be insufficiently

powered. We determined therefore to use higher doses of BrdU in subsequent experiments (see below)

4.2.4 Conclusion

This study has shown that, under the present dosing protocol, LY404187 was unable to induce significant synaptogenesis in the molecular layer of the DG and alter the rate of neurogenesis. Although the study does not support my initial hypothesis the study was carried out correctly and still addresses important issues pertaining to the ability of AMPA potentiators to induce *in vivo* structural plasticity that are worthy of further investigation.

4.3 Biochemical changes associated with AMPA potentiation

4.3.1 Summary

The studies described in Chapter 3.3 were aimed at investigating the ability of the AMPA potentiator LY450108 (0.5mg/kg) to induce persistent changes in a number of relevant biochemical parameters. Each study had three groups of animals. The first group received LY450108 (0.5mg/kg) twice daily for three days, the second for ten days and the final group received ten days of compound and seven days of vehicle doses. All groups included vehicle-dosed controls. The studies all reveal little or no change in the measured end points across time points and treatment groups. There was no significant change in the levels of phosphorylated ERK1/2, CREB, the rate of neurogenesis of neuronal development and the levels of the principal monoaminergic neurotransmitters across multiple brain regions. There are a number of reasons for this outcome, insufficient dosing of LY450108, lack of sensitivity of the techniques and an inaccurate hypothesis.

4.3.2 Adequacy of LY450108 treatment regimen

The dose of LY450108 used in the studies (0.5mg/kg) was selected because of the efficacy seen at a similar dose with AMPA potentiators from the same family as LY450108 in a number of *in vivo* models. LY450108 has been shown to increase BDNF protein and mRNA in cortical neurons and potentiate synapses at comparable concentrations to LY404187 *in vitro* (V. Lakics 2006). Histological and behavioural studies in rats following 6-OHDA lesions of the cortico-striatal tracts show that

LY450108 (0.5mg/kg) induces significant recovery in both endpoints with comparable potency to LY404108 and LY540430 (Murray, Whalley et al. 2003). Other AMPA potentiators of the same class have been shown to be active in animal models of depression (Li, Tizzano et al. 2001), induce BDNF expression *in vivo* following acute and chronic exposure (Mackowiak, O'Neill et al. 2002) and promote structural recovery *in vivo* at the same dose of 0.5 mg/kg (Quirk and Nisenbaum 2002; O'Neill, Murray et al. 2004). However all these studies were carried out in rats and currently there is no available data on the pharmacokinetics of LY450108 in mice. It is possible therefore that the brain availability of LY450108 in these studies is significantly different from those seen in rats (MJ O'Neill personal communication). The activity of AMPA potentiators *in vivo* has a pronounced bell shaped dose response curve. In fact, although 0.5mg/kg of LY404187's active isomer LY451646 increased BDNF and Trk-B mRNA in the hippocampus following seven days dosing, higher doses were ineffective and lower doses caused a decrease in BDNF and Trk-B expression (Mackowiak, O'Neill et al. 2002).

4.3.3 Methodology

If LY450108 0.5mg/kg is a high enough dose to penetrate the brain in sufficient concentration then the temporal aspect of experimental design must be reviewed. The design was aimed at identifying whether AMPA potentiator administration could induce long term plastic changes in a number of biophysical measures. There is limited data regarding the long term effects of AMPA potentiators *in vivo*. The principal evidence for choosing the time course of the studies monitoring the histological and behavioural

improvements associated with AMPA potentiation was from data obtained with an animal model of Parkinson's disease (MJ O'Neill, personal communication). What these studies consistently reveal is that behavioural recovery, as measured by the rate of turning following apomorphine administration, is only apparent following approximately ten days administration of AMPA potentiator (Murray, Whalley et al. 2003). Other studies using CX516, show that the improved performance in a short term memory task following 10 days administration have been shown to persist for 7 days following withdrawal of treatment (Hampson, Rogers et al. 1998; Hampson, Rogers et al. 1998). The choice of 3, 10 and 17 days time points were chosen to identify changes that had not occurred following 3 days administration but had developed by 10 days and showed some evidence of persisting 7 days following withdrawal of treatment.

If LY450108 at 0.5mg/kg is sufficient to produce prolonged potentiation of central glutamatergic synapses triggering multiple downstream cascades, then there is a possibility the techniques used to assess the different endpoints may not be appropriate or sensitive enough to detect these changes.

Western blotting techniques are excellent measures of large on/off changes in protein expression. They are among the most widely used techniques in biology but are not ideal for revealing subtle changes in protein levels. They are semi-quantative techniques and do not generate raw data that is amenable to standard statistical evaluation. No change was detected in the levels of phosphorylated ERK 1/2 when assessed using Western blots. It is possible that Western blotting is not sensitive enough to detect a change in expression that is likely to be subtle (see below) and restricted to certain cell types within the hippocampus. For this reason a second measure of protein

expression using immunohistochemistry was incorporated into the experimental design. This technique holds a number of advantages over standard Western blotting. First it allows accurate identification of individual positive cells while Western blots were carried out on protein extracted from the whole hippocampus. Using the protocol described, only a percentage of cells were stained positive for pERK and pCREB which allowed for a more quantitative approach as individual positive cells can be counted. The experiment was designed with two parallel end points to give support to any positive findings but also to ensure the largest chance of detecting a change in expression.

Peripheral administration of the uridine analogue BrdU is an established way to measure neurogenesis *in vivo* (Gage, Kempermann et al. 1998; Kempermann and Gage 2000). The BrdU dose we used (2x 100mg/kg doses 24hours prior to cull) was larger than that used in many other studies because it gives a larger number of positive cells in the thin section used in this study (Gould and Gross 2002). The double labelling of newly generated cells with other markers is also a commonly used technique. There are a range of neuronal markers that can be used to identify cells at multiple stages of development including NeuN, Doublecortin and Nestin. The neuronal marker NeuN however remains the most widely used (Kempermann, Wiskott et al. 2004). In this study, double labelled cells were often identified in the granular layer of the hippocampus showing that the cells had migrated and were potentially being integrated into the hippocampal circuitry (Kempermann and Gage 2000; van Praag, Schinder et al. 2002). The identification of newly divided cells in the hippocampus by this technique is well established and is amenable to accurate quantification. As such I am satisfied the

results give an accurate reflection of both the rate of neurogenesis and neuronal development.

HPLC allows rapid separation and accurate quantitative resolution of levels of the components of a mixture. In this case the technique was used to measure levels of the principal aminergic neurotransmitters and their main metabolites. The electrochemical detection system used is highly sensitive and can allow the detection of picograms of the desired compounds. The reproducibility and reliability of the technique is borne out not only by tight distribution of the data but by the similarity with results from other groups. Levels obtained for DA (~15ng/mg) in the striatum, 5HT (~0.5ng/mg) and NA (~0.4ng/mg) in the hippocampus and NA (~0.5ng/mg) in the neo cortex are almost identical to those found in a recent study (Chourbaji, Hellweg et al. 2004).

4.3.4 Discussion

The intellectual basis of this study was the neurotrophin hypothesis of depression and the potential benefit AMPA potentiators may have in the treatment of this condition (Duman, Heninger et al. 1997; Manji, Quiroz et al. 2003; Castren 2005; Alt, Nisenbaum et al. 2006). Recent basic and clinical studies have implicated the neurotrophin BDNF and its receptor Trk-B in mediating dendritic structural integrity in the hippocampus and neurogenesis, both of which are believed to be reduced in depressed patients and are manipulated by common antidepressants (Manji, Quiroz et al. 2003; Peled 2005). Recent work by O'Neill and colleagues has suggested that AMPA potentiators may be able to promote structural plasticity *in vivo* and LY404187 has been shown to enhance

the rate of neurogenesis (Bai, Bergeron et al. 2003). AMPA potentiators have also been shown to be active in animal models of depression (Bai, Li et al. 2001; Quirk and Nisenbaum 2002). The study was aimed at identifying the effects of LY450108 on two potential indicators of antidepressant properties: the rate of neurogenesis and the levels of monoaminergic neurotransmitters across the brain (Bunney and Davis 1965; Schildkraut 1965; Brown and Gershon 1993; D'Sa and Duman 2002; Santarelli, Saxe et al. 2003).

BDNF expression is regulated by AMPA receptors through the activation of ERK 1/2 and the transcription factor CREB (see above section 4.1.3) (Finkbeiner, Tavazoie et al. 1997; Shieh, Hu et al. 1998; Tao, Finkbeiner et al. 1998). Although AMPA potentiators have been hypothesised to act though MAPK and CREB *in vivo* (O'Neill, Bleakman et al. 2004) this has yet to be demonstrated. The data in this study show no significant change in the level of phosphorylation of either of these proteins. One reason for this could be that the animals were culled 18 hours following the final dose. Levels of other biarylpropylsulphonamides typically have a short half-life, plasma levels returning to baseline levels within 18 hours (O'Neill, Murray et al. 2005) and it is likely that any direct activation of MAPK and CREB would be over by this time. The reason for this delay was to identify enhanced expression due to the action of BDNF.

Rates of neurogenesis have been shown to be significantly altered by chronic antidepressant administration (Malberg, Eisch et al. 2000) and by both acute and chronic administration of AMPA receptor potentiator (Bai, Bergeron et al. 2003). Levels of neurogenesis were unaltered across treatment groups and time points in this study. Given the sensitivity of the method and the previous demonstration of an enhancement of

neurogenesis by an AMPA potentiator it is likely that the main cause of a lack of detectable change is a problem of insufficient dosage.

The most commonly prescribed antidepressants inhibit the re-uptake of aminergic neurotrasmitters (mainly 5-HT but also NA and DA) (Nestler, Barrot et al. 2002). If LY450108 had been shown to enhance levels of monoamines, this would have provided support for their potential as effective antidepressants. However the lack of any effect as shown in this study does not preclude a possible benefit in this condition. Firstly SSRIs and SNRIs act to enhance synaptic levels of neurotransmitter, while in the study the total levels have been measured. It is possible that AMPA receptor potentiation may modulate the release of neurotransmitter. Secondly the neurotrophin hypothesis of depression places the role of CREB and BDNF downstream of antidepressants (Duman et al 2000). AMPA potentiators, able to directly enhance levels of BDNF, following acute administration, may be able to bypass mechanisms by which common antidepressants work, possibly even bypassing the therapeutic lag associated with SSRIs.

4.3.5 Conclusions

In the absence of extensive pharmacokinetic data for LY450108 in mice it cannot be concluded with any certainty that this experiment has yielded neutral results due to insufficient dosing. However, given the well established *in vivo* actions of other biarylpropylsulphonamides in a range of biochemical, behavioural and physiological paradigms in multiple species it is likely that this is the case. It might be expected that even with a suboptimal dose, repeated exposure to AMPA potentiator may induce

measurable effects in one of the study's endpoints. However due to the indirect measures used in this study and the fact that the animals were not all normal healthy adults it is possible that any effect would be negligible.

Despite the obvious disappointment with the results the issues raised in this study; the persistence of AMPA potentiator action and the potential for inducing forms of plasticity relevant to novel therapeutic mechanisms for the treatment of depression, are very important and are worthy of further investigation.

4.4 General Discussion

4.4.1 Thesis Review

The evidence presented in this thesis suggests that AMPA potentiation can induce structural plasticity *in vitro* following 3 days exposure to LY404187. However much of the study has yielded inconclusive results. By its very nature AMPA potentiation does not induce large, dramatic developmental and neurotrophic changes in cells. Long-term changes induced by AMPA receptors are likely to be due to the downstream actions of neurotrophins up-regulated by repeated AMPA receptor activation (Limatola 2004; O'Neill, Bleakman et al. 2004). The multiple effects of BDNF and other neurotrophins are thus diluted temporally and topographically and it is understandable that the *in vivo* studies yielded neutral results. The evidence showing neurotrophin-mediated mechanisms of structural development *in vitro* (Voss, Milne et al. 2006) is not easily transferred to an *in vivo* system. The magnitude of the effect seen *in vitro* was relatively small and was detectable only through well powered and statistically sound analysis. Extrapolating these data to an *in vivo* system, with its increased inherent variability and the multiple cell types, systems and different biochemical environment has proved unsuccessful. There is also data suggesting that BDNF expression is refractory followed repeated exposure to AMPA potentiators (Lauterborn, Truong et al. 2003). However, data using biarylpropylsulphonamides has repeatedly shown that they can have biological effects following chronic exposure (Mackowiak, O'Neill et al. 2002; Bai, Bergeron et al. 2003).

There is the strong possibility that the chosen dose of 0.5mg/kg of LY450108 was insufficient to reach adequate concentrations in the brain of mice. Unfortunately there was neither the time nor the technical expertise to develop a full pharmacokinetic profile for LY450108 in mice and the dose was chosen from a large body of literature describing the effects of other AMPA potentiators in a range of pre-clinical models.

Despite the disappointment of neutral results I believe the experiments in this thesis were consistently well designed and executed. The demonstration that LY404187 can induce structural plasticity *in vitro* represents a novel observation with important implications for the potential therapeutic applications of AMPA potentiators.

4.4.2 Recent advances in compound development

Two reports have recently appeared in the literature describing new generations of positive AMPA modulators and cognitive enhancers (Romanelli, Galeotti et al. 2006; Zarrinmayeh, Tromiczak et al. 2006). Romanelli et al. reported the development of aniracetam derivatives that exhibit potencies 4 orders of magnitude higher than traditional AMPAkines. As well as facilitating synaptic transmission they improved performance in memory tasks and enhanced acetylcholine release in rat hippocampal slices; data that holds obvious promise in the treatment of Alzheimer's disease and other cognitive disorders. Zarrinmayeh et al reported the generation of a whole family of novel AMPA receptors potentiators, the most potent of which, LY2059346, displayed low nanomolar EC_{50} at recombinant GluR1-4 subunits.

4.4.3 Clinical trials

The absence of clinical data on the efficacy of AMPA potentiators in treating neurological conditions and cognitive impairment means that it is too early to judge the success of AMPA potentiator development. AMPA potentiation is being studied principally for the development of clinically effective drugs. Therefore it is clear that the future of AMPA potentiators lies in the clinic rather than the laboratory. Following the publication of a number of reports describing the early development and key properties of a series of AMPA potentiators, there has been fewer recent reports regarding their development. Currently there are a number of clinical trials for the use of AMPA potentiators in neurological conditions. Organon currently has compounds in both Phase I and Phase II trials for the treatment of schizophrenia and mild cognitive impairment (website 2006). Servier Pharmaceuticals have an AMPA modulator S18986 (Lebrun, Pilliere et al. 2000) in Phase II clinical trials (clinical gov.net). Cortex pharmaceuticals, the company set up by Gary Lynch to develop clinical AMPA potentiators, recently had one of it's compounds CX717 (Porrino, Daunais et al. 2005) put on clinical hold by the FDA following concerns regarding the toxicity of the compound. This hold has recently been lifted prompting a resumption of clinical trials (Cortex pharmaceuticals web site 2006). Lilly Neuroscience's compounds LY450108 and LY451395 have both passed Phase I studies (Jhee, Chappell et al. 2006).

4.4.4 Future directions

The primary concern regarding the development of AMPA potentiators is their selectivity. Early publications contained results of in depth screening at a variety of

receptor subtypes and splice variants. Recent generations of AMPA potentiators show a wide range of selectivity and affinity across all GluR subtypes (Zarrinmayeh, Tromiczak et al. 2006). This pool of compounds, coupled with a more sophisticated knowledge of the receptors distribution across different areas of the brain could allow the development of a range of disease-tailored compounds. Compounds could be developed specifically for cognitive enhancement, working on the hippocampus to facilitate the formation of long term memories, or a compound working specifically in the basal ganglia to promote neurotrophic effects in Parkinson's disease or for the limbic system in mood disorders. One important caveat with this, as with all notions of tailor made drugs is that as the costs associated with the development of multiple compounds increases the number of patients that will benefit from each drug is reduced.

4.4.5 Conclusions

The positive modulation of AMPA receptors has been shown to facilitate the formation of memories, enhance the rate of neurogenesis, induce the expression of BDNF and promote structural plasticity in pre-clinical models. These features make AMPA potentiators clinically relevant in a number of conditions that display deficits in one or more of these areas.

AMPA potentiators have been developed from first principals to clinical trials in little over a decade. The challenge of the next ten years is to realise the undoubted potential of these compounds to treat some of the most common neurological conditions.

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Molecular mechanisms of neurite growth with AMPA receptor potentiation

Oliver P. Voss [a,*], Stuart Milne [a,c], John Sharkey [a,c], Michael J. O'Neill [b], James McCulloch [a,c]

[a] *Division of Neuroscience, The University of Edinburgh, The Chancellor's Building (Room FU.203), 49 Little France Crescent, Edinburgh EH16 4SB, UK*
[b] *Eli Lilly & Co. Ltd. Lilly Research Centre, Windlesham, Surrey GU20 6PH, UK*
[c] *Astellas CNS Research in Edinburgh, The University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, UK*

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Abstract

Positive allosteric modulation of AMPA receptor function has therapeutic potential in a number of psychiatric disorders and neurodegenerative diseases. AMPA receptor potentiators can induce neurite sprouting in vivo. Using a strategy of combined morphological and biochemical analyses, we investigated the effect of the AMPA receptor potentiator LY404187 on neurite growth in the SH-SY5Y human neuroblastoma cell line. LY404187 (0.1−10 μM) increased average neurite length and neurofilament expression when co-administered with s-AMPA. Co-incubation with s-AMPA and LY404187 also increased Trk receptor expression. All actions of LY404187 were sensitive to AMPA receptor blockade by the selective antagonist CNQX (10 μM). Antibody sequestration of BDNF attenuated neurite growth following AMPA receptor potentiator administration, suggesting that LY404187 increases neurite length in vitro by a BDNF mediated mechanism. AMPA receptor potentiation activates multiple intracellular neurochemical cascades and the present report identifies BDNF as one key mediator of the neurotrophic effects of AMPA receptor potentiation.

Keywords: LY404187; AMPA potentiators; BDNF; Neuroplasticity

1. Introduction

Glutamate receptor dysfunction has been implicated in a variety of neurological disorders including depression, schizophrenia and Parkinson's Disease (Chase and Oh, 2000; Paul and Skolnick, 2003; Tuominen et al., 2005). AMPA receptors mediate the majority of excitatory neurotransmission at central mammalian synapses (Hollmann and Heinemann, 1994) and regulate many developmental, neurotrophic and cognitive processes (Collingridge and Singer, 1990; Limatola, 2004). The modulation of AMPA receptor activity is thus being actively investigated as a possible treatment for a variety of cognitive and neurodegenerative conditions (Lynch, 2004; O'Neill et al., 2004a). Due to the rapid desensitisation of AMPA receptors (Dingledine et al., 1999) and their involvement in excitotoxic cell death (Choi, 1992) direct AMPA receptor agonists are not desirable pharmaceutical agents. Consequently a number of compounds, which act as positive allosteric modulators, with no intrinsic agonist activity, have been developed (Larson et al., 1995; Arai et al., 1996; Sekiguchi et al., 1997; Ornstein et al., 2000; Buccafusco et al., 2004). These compounds have been shown to enhance synaptic transmission in vitro and in vivo (Gates et al., 2001; Vandergriff et al., 2001), facilitate the induction of LTP (Staubli et al., 1992; Arai et al., 2004), increase BDNF expression in vivo and in vitro (Legutko et al., 2001; Mackowiak et al., 2002; Lauterborn et al., 2003) and promote memory formation in animals and in humans (Staubli et al., 1994; Granger et al., 1996; Ingvar et al., 1997; Buccafusco et al., 2004).

* Corresponding author. Tel.: +44 (0) 131 242 6181; fax: +44 (0) 131 242 9371.
E-mail address: o.p.voss@sms.ed.ac.uk (O.P. Voss).

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The biarylpropylsulphonamide family of AMPA receptor potentiators (Ornstein et al., 2000) have recently been shown to be beneficial in a rodent model of Parkinson's Disease, demonstrating histological and behavioural improvements (O'Neill et al., 2004b, 2005). In this study the authors concluded that enhanced survival and sprouting of dopaminergic cells mediated these benefits and it has been hypothesised that enhanced central levels of BDNF underlie this form of structural plasticity (O'Neill et al., 2004a).

In the present study we set out to test the hypothesis that AMPA receptor potentiation could induce morphological plasticity. Using a sensitive neurite growth assay, we demonstrate that the AMPA receptor potentiator LY404187 (Quirk and Nisenbaum, 2002) significantly increases neurite length and expression of the cytoskeletal protein neurofilament in the SH-SY5Y human neuroblastoma cell line and that this increase is mediated by the neurotrophin BDNF. Our data support the hypothesis that compounds which are able to enhance central levels of neurotrophins, such as AMPA potentiators, are able to promote structural plasticity and may be useful in the treatment of neurological disorders.

2. Methods

All reagents were purchased from Sigma (Poole, UK), unless otherwise stated.

2.1. Cell culture

SH-SY5Y cells (ECACC No. 94030304) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated foetal calf serum (FCS) (Gibco), 2 mM L-glutamine and 1% penicillin/streptomycin mix. Cells were cultured for up to 6 weeks (~passage 20 in accordance with ECACC guidelines). In neurite growth experiments, cells were plated in NUNC 6-well dishes at a density of 1×10^5 cells/well 24 h prior to testing. Medium was replaced with fresh DMEM (2% FCS, 2 mM L-glutamine) and drugs applied for 72 h. In Western blot experiments cells were seeded at a density of 1×10^6 per 9 cm dish and again incubated in DMEM (2% FCS, 2 mM L-glutamine) containing drug treatment for 72 h. Cells were treated with s-AMPA, LY404187 (Eli Lilly), CNQX and Ab-BDNF (1:200, N-20, sc-546, Santa Cruz) or vehicle (1:10,000 Ethanol). All drugs were stored at −20 °C in 10 mM stock aliquots and fresh dilutions were made on each experimental day.

2.2. Neurite growth assay

Cells were fixed with ice-cold 4% paraformaldehyde in Phosphate Buffered Saline (PBS) for 2 h at 4 °C, washed and stored in PBS. Phase-contrast images (200× magnification) were obtained from each well (Leitz Laborlux microscope with Polaroid CCD camera running Polaroid DMC2 software). The experimenter, blinded to treatment group, selected fields at random. Digitised images of dispersed, neurite bearing cells were captured and morphometric analysis was performed using Image J software (NIH). The system was checked for image compression and calibrated before use by taking horizontal and vertical images (200×) of a standard graticule. Neurites were traced manually and the length calculated. All process shorter than 20 μm (average width of cell body) were not included in further analysis. For all groups, unless otherwise specified, between 300 and 450 neurites were collected from 10 separate wells over 4 experimental days.

As the data were not distributed normally, all measurements of neurite length were analysed by Kruskall–Wallis analysis of variance on ranks, followed by comparison to vehicle using Dunn's method. In all cases statistical significance was set at $p < 0.05$. For BDNF antibody and CNQX studies additional, multiple Mann–Whitney comparisons were run as a post-hoc test following analysis of variance. In this study p values were corrected for multiple comparisons using the Bonferroni procedure.

2.3. Western blotting

After 72 h incubation, SH-SY5Y cells were washed with ice-cold PBS and lysed in 500 μl NP40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl pH 7.4; 10 mM EDTA; 0.6% Nonidet P40; 1 mM Na_3VO_4; 10% glycerol; 10 μg/ml pepstatin; 1 mM phenyl methyl sulfonyl fluoride). Cell lysates were incubated on ice for 30 min before removing cell debris by centrifugation (15,300 g/10 min/4 °C) and protein concentrations determined using a modified Lowry method (Bio-Rad D2 Protein Assay kit; Bio-Rad Laboratories, Hemel Hempstead, UK). In Western blot experiments, 10 μg of protein in standard loading buffer (25 mM Tris–HCl (pH 6.8); 0.8% SDS; 1% 2-mercaptoethanol; 4% glycerol; 0.01% bromophenol blue) was loaded on a 4–12% Tris glycine gel and separated by SDS-PAGE (200 mV for 45 min). Proteins were transferred to a PVDF membrane (Millipore (UK) Ltd, Watford, UK), and membrane incubated in Tris Buffered Saline (TBS)-Tween (50 mM Tris–HCl; 150 mM NaCl; 0.05% v/v Tween-20) containing 5% albumin w/v for 1 h. Sequentially membranes were probed with primary antibody; neurofilament (1:2000 anti-200 kDa Neurofilament Heavy – Ab7795, Abcam), Trk (1:500, B-3 sc-7268, SantaCruz), β-actin (1:5000, Sigma, UK) and peroxidase-conjugated IgG (1:30,000; Sigma, UK) and then detected with Enhanced Chemiluminescence Detection system (ECL plus; Amersham Biosciences UK Ltd., Little Chalfont, UK). Between each step membranes were washed three times for 5 min with TBS-Tween. Chemiflourescence levels were corrected for background and normalised to levels for control cells. Data were analysed by Kruskal–Wallis analysis of variance on Ranks with multiple comparisons using Dunn's method. In all cases significance was set at $p < 0.05$.

3. Results

3.1. Neurite growth assay

Power calculations revealed that approximately 350 neurites were required to detect a 5 μm increase in neurite length using a power of 80% and p value of 0.05.

3.2. AMPA potentiation increases neurite length and neurofilament expression

As an assessment of structural plasticity, neurite growth in the human neuroblastoma line SH-SY5Y was measured (Fig. 1a). Cells exposed to s-AMPA (0.1–10 μM) alone exhibited no significant change in neurite length (Fig. 2 *left*). Pilot studies confirmed that higher concentrations of s-AMPA (100 μM) induced cell death (data not shown). LY404187 (0.1–10 μM) alone failed to produce any significant increase in average neurite length over the concentration range examined, although a decrease in neurite length was observed at 10 μM (Fig. 2 *centre*). However, when co-administered with s-AMPA (0.1 μM), LY404187 (0.1–1 μM) produced a significant, concentration dependant increase in neurite length (Fig. 2 *right*). Frequency distribution analysis reveals that the shift in neurite distribution was more pronounced in shorter neurites, explaining the numerically subtle increases in average neurite length (Fig. 1b). AMPA potentiator-induced neurite growth was sensitive to AMPA receptor blockade as it was significantly blocked by co-incubation with CNQX (10 μM) (Fig. 3).

The increase in average neurite length induced by AMPA and LY404187, was compared to that induced by the classical

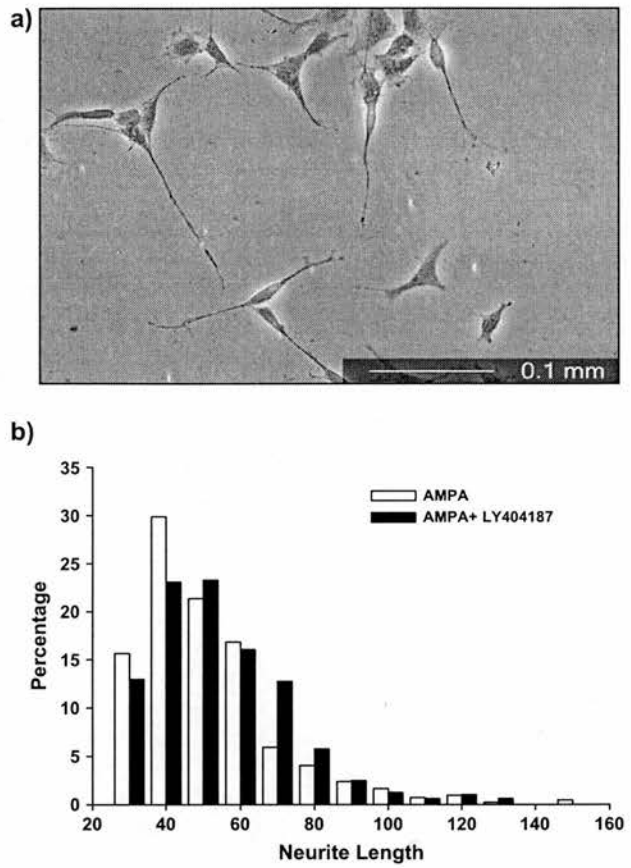

Fig. 1. Measuring neurite growth in vitro. a) Following 72 h incubation each SH-SY5Y cell develops 2–3 neuritic processes. 64 fields containing approximately 200 neurite bearing cells were acquired for each treatment (approximately 400 neurites per treatment) and used for morphological analysis (Scale bar 100 μm). b) Frequency distribution analysis of the length of neurites. The distribution profile of neurites following incubation with vehicle, AMPA and LY404187 alone were similar. Incubation with LY404187 (0.1 μM) together with AMPA (0.1 μM) produced a significant increase in neurite length and this was reflected by an increase in the median length (41.8 μm to 46.3 μm), reflecting a reduction in neurites 20–40 μm and an increase in neurites between 40 μm–80 μm. There was no change in the percentage of neurites measuring above 80 μm. Data represents >400 neurites collected over 4 independent experimental days.

differentiating factors BDNF (10 ng/ml), NGF (10 ng/ml) and retinoic acid (RA 100 nM) (Fig. 4). Because each compound was prepared in a different vehicle, relative increases were used to assess neurite growth. Co-incubation of AMPA and LY404187 induced approximately a 10% increase in neurite length which was comparable to that produced by BDNF. NGF and RA increased average neurite length by around 20–30%.

As an alternative, surrogate marker for neuritic development and growth western blots for neurofilament heavy chain (NF-H) were performed. LY404187 (0.1 μM) produced occasional, small increases in NF-H expression that were blocked by CNQX (data not shown). These effects were likely due to the presence of low levels of glutamate in the media following 72 h incubation. Co-treatment with LY404187 (0.1 μM) and s-AMPA (0.1 μM) however induced robust increases in immunoreactivity for NF-H (Veh = 0.75 ± 0.2 SEM, A + LY: 2.7 ± 0.6 SEM) that were significantly blocked by the addition of the AMPA receptor blocker CNQX (10 μM) (CNQX + AMPA + LY = 0.4 ± 0.2) (Fig. 5).

Taken together these data strongly indicate significant morphological plasticity induced by AMPA receptor potentiation.

3.3. AMPA potentiation enhances Trk receptor expression

Neurotrophins have been strongly implicated as mediators of the beneficial effects seen with administration of AMPA potentiators. As an indicator of enhanced neurotrophic activity Trk receptor expression was next monitored by western blotting. Co-incubation with AMPA (0.1 μM) and LY404187 (0.1 μM) produces robust increases in immunoreactivity, (Veh = 0.8 ± 0.1 A + LY = 3.8 ± 0.8 SEM), which are significantly blocked by co-incubation with CNQX (10 μM) (CNQX + AMPA + LY = 0.57 ± 0.3) (Fig. 6).

3.4. Antibody sequestration of BDNF blocks increase in neurite length following AMPA potentiation with LY404187

BDNF is the main neurotrophin thought to mediate the actions of AMPA potentiators. The role of BDNF as a mediator

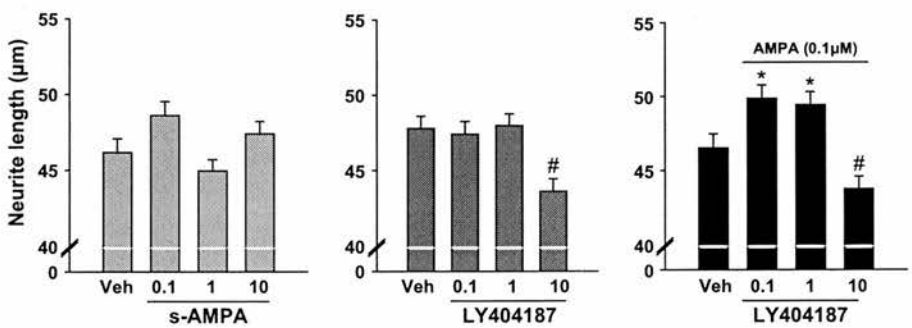

Fig. 2. AMPA potentiation increases neurite length. Incubation for 72 h with either (left) s-AMPA (0.1–10 μM) or (centre) LY404187 (0.1–10 μM) alone produced no significant change in neurite length. Co-administration of s-AMPA (0.1 μM) and the AMPA potentiator LY404187 (0.1–1 μM) however, significantly increased average neurite length (right). Data are presented as the mean neurite length ± SEM of 400–500 neurites for each treatment. (Veh = vehicle *,# = $p < 0.05$.)

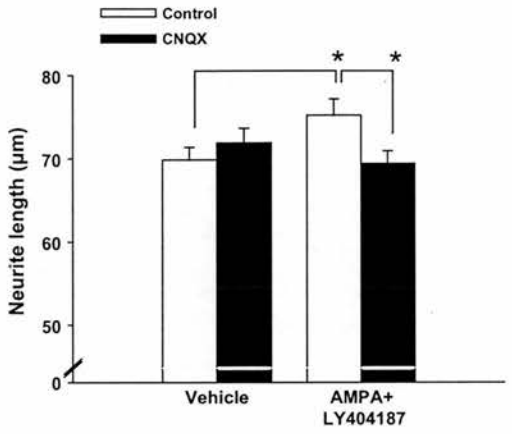

Fig. 3. AMPA receptor blockade attenuates increase in neurite length. Incubation of SH-SY5Y with CNQX (10 µM) attenuated the increase in neurite length following co-incubation with s-AMPA (0.1 µM) and LY404187 (0.1 µM). Data are presented as the mean average length ± SEM of 300–400 neurites. * = $p < 0.05$.

of neurite growth was investigated. The addition of BDNF antibody (1 µg/ml) attenuated the increase in average neurite length induced by co-administration of s-AMPA (0.1 µM) and LY404187 (0.1 µM) (Fig. 7). Importantly, 72 h incubation with another rabbit-raised antibody (anti-Rat IgG) did not inhibit the increase in average neurite length induced by AMPA potentiation that the effect of the anti-BDNF antibody was specific to the sequestration of BDNF and not a non-specific inhibition of neurite extension (data not shown).

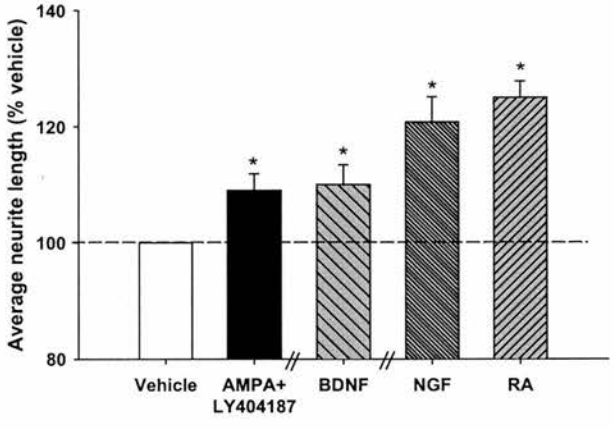

Fig. 4. Comparison between AMPA potentiation and neurotrophins. BDNF (10 ng/ml), NGF (10 ng/ml) and retinoic acid (RA) (0.1 µM) treatment are compared to AMPA (0.1 µM) + LY404187 (0.1 µM) (A + LY) by their ability to enhance neurite length. Graphs represent average percentage increase ± SEM of 4 independent experiments. Data are expressed as percentage change from multiple vehicle treatments (appropriate for each compound). Individual values AMPA + LY: (veh = 65.9 µm ± 2.1, A + LY = 71.9 µm ± 1.5) BDNF: (veh = 64.2 µm ± 1.7, BDNF = 75.5 µm ± 2.2) NGF: (veh = 48.3 µm ± 3.1, NGF = 58.6 µm ± 1.9) RA: (veh = 52.3 µm ± 3.3, RA = 66.7 µm ± 2.3). Statistical significance was assessed by Student *t*-tests on the average lengths from each experiment * = $p < 0.05$. Graph shows the distribution of >400 neurites collected from 10 wells from 5 independent experiments.

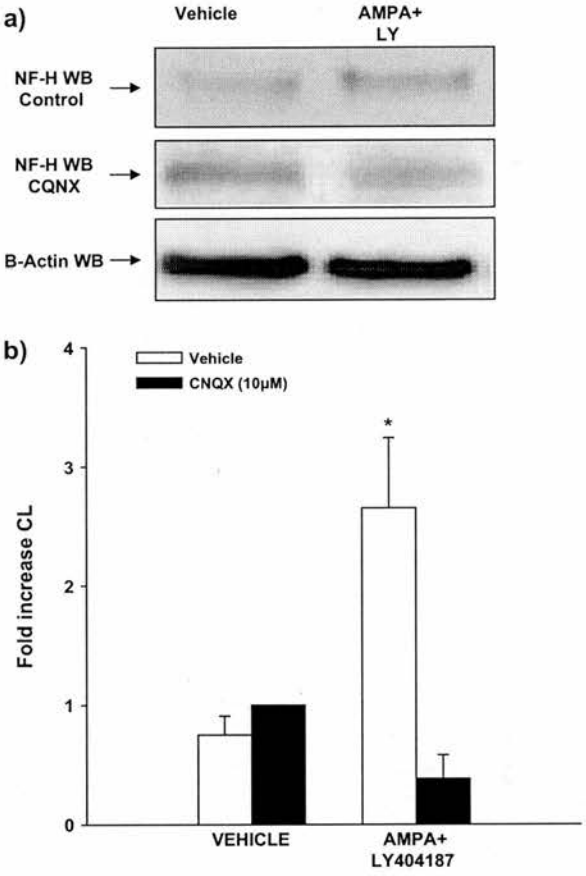

Fig. 5. AMPA receptor potentiation increases neurofilament expression. (a) Representative Western blots showing neurofilament heavy chain expression levels from control (upper) and CNQX (10 µM) treated cells (middle). Equal loading was confirmed by blotting for β-actin (lower). AMPA (0.1 µM) + LY404187. (b) Mean fold increase in chemiluminescence normalised to Vehicle + CNQX treated cells ± SEM of 4 independent experiments. * = $p < 0.05$.

4. Discussion

AMPA receptor potentiators have been shown to act as cognitive enhancers in a variety of animal and human paradigms (Black, 2005), and have been hypothesised to act by facilitating the induction of LTP (Lynch, 2004). However, more recent studies suggest that AMPA potentiation may have neurotrophic and neuroprotective properties (Dicou et al., 2003; O'Neill et al., 2004b, 2005). In this study we aimed to directly test the hypothesis that AMPA potentiation can induce structural plasticity. Our findings demonstrate that direct application of an AMPA receptor potentiator can induce neurite growth in SH-SY5Ys that is attenuated by AMPA receptor blockade. We showed that this increase is comparable to that induced by the more traditional neurotrophic agents BDNF, NGF and retinoic acid. As an alternative measure of plasticity we demonstrated that the expression of the cytoskeletal protein neurofilament is enhanced in an AMPA receptor mediated fashion following AMPA potentiation. We then showed that Trk receptor expression is also enhanced, implicating the actions of

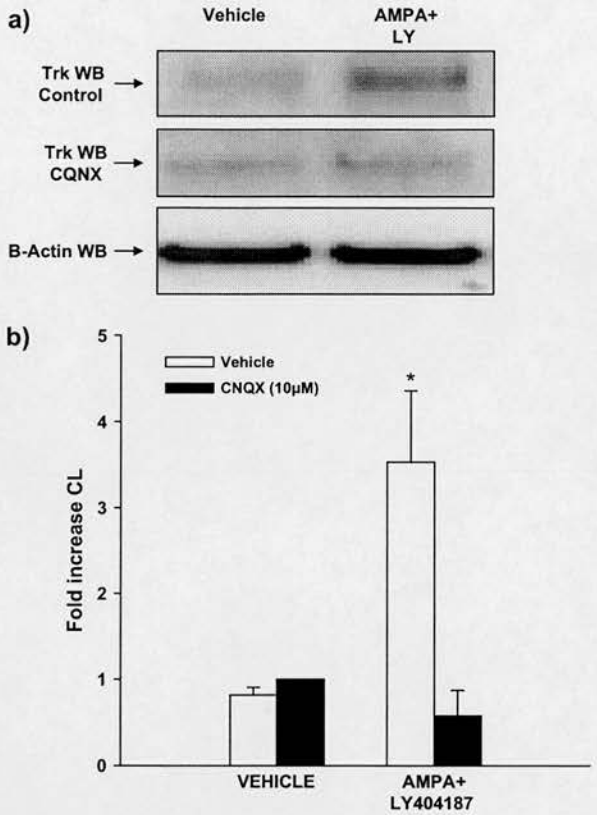

Fig. 6. AMPA receptor potentiation increases Trk receptor expression. (a) Representative Western blots showing Trk receptor expression levels from control (upper) and CNQX (10 μM) (middle) treated cells. Equal loading was confirmed by blotting for β-actin (lower). (b) Mean fold increase normalised to Vehicle + CNQX treated cells ± SEM of 4 independent experiments. * = $p < 0.05$.

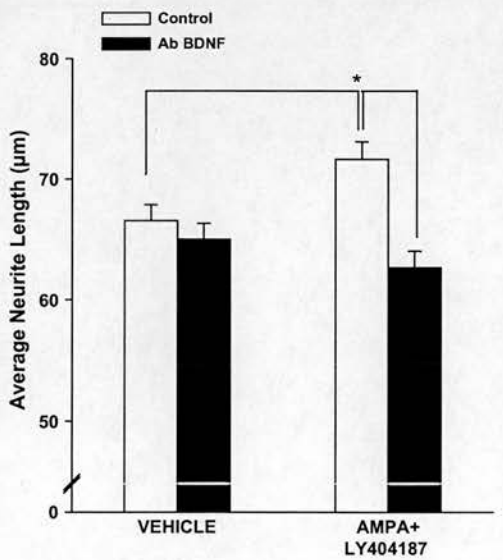

Fig. 7. Antibody sequestration of BDNF attenuates increase in neurite length. Co-incubation with anti-BDNF mouse IgG (Ab BDNF) (1 μg/ml) attenuated neurite outgrowth following co-incubation with s-AMPA (0.1 μM) and LY404187 (0.1 μM). Data are presented as mean neurite length of 400–500 neurites ± SEM. * = $p < 0.05$.

neurotrophins. We finally showed that neurite growth promoted by AMPA potentiation is dependent of BDNF. Our results support our initial hypothesis and shed light into the mechanisms of AMPA potentiator mediated effects.

Enhancing central plasticity, neuritic sprouting and regeneration are promising therapeutic strategies for the treatment of a number of neurological conditions (Manji et al., 2003; Fernandez-Espejo, 2004; Mueller et al., 2005). Reductions in volume and structural abnormalities have been observed in a number of brain regions in humans with depression (Sheline, 2003). Reduced rates of neurogenesis (D'Sa and Duman, 2002), decreased levels of BDNF (Angelucci et al., 2005) and plasticity imbalances (Peled, 2005) have all been implicated. Neuroprotective and neurotrophic strategies are also being investigated for the treatment for Parkinson's disease, focusing on the actions of the neurotrophins BDNF and GDNF (Mamounas et al., 1995; Gash et al., 1998; Fernandez-Espejo, 2004). Compounds that are able to enhance central levels of neurotrophins and promote structural plasticity and regeneration would be well placed as potential therapeutic agents in each of these conditions (O'Neill et al., 2004a). Biarylproposulphonamides have been demonstrated to enhance neurogenesis (Bai et al., 2003), BDNF expression in vivo (Mackowiak et al., 2002), sprouting of nigro-striatal dopamingergic terminals and show behavioural effects in animal models of depression (Li et al., 2001) and Parkinson's disease (O'Neill et al., 2004b, 2005). Our data extend these findings, demonstrating the ability of AMPA receptor potentiation to enhance neuritic growth in vitro, supporting the notion that LY404187, and related compounds have favourable profiles for the treatment of a range of neurological conditions.

SH-SY5Y cells possess biochemical characteristics similar to dopaminergic cells, such as tyrosine-β-hydroxylase activity (Oyarce and Fleming, 1991) and are a common model for the investigation of neuronal differentiation and neurite growth (Ivankovic-Dikic et al., 2000; Cui et al., 2003; Price et al., 2003; Jamsa et al., 2004; Daniel et al., 2005). Our results demonstrate that LY404187 in the presence of s-AMPA induced neurite outgrowth in SH-SY5Y cells. The magnitude of the effect was comparable to that induced by BDNF incubation and around half that induced by NGF and retinoic acid. LY404187 thus compares favourably to classical neurite growth inducers, especially when considering that an allosteric modulator of an ion channel is being compared to neurotrophins and a powerful differentiation factor. This growth was dependent on AMPA receptors, as incubation with CNQX, a competitive AMPA receptor antagonist (Drejer and Honore, 1988) attenuated the response. The EC_{50} of LY404187 ranges from 0.15 μM at recombinant GluR2 receptors (Miu et al., 2001) and 1.3 μM for native AMPA receptors on isolated cortical rat neurons (Quirk and Nisenbaum, 2002). At >10× these concentrations LY404187 reduced the average length of neurites. This was most likely due to a non-specific effect of the compound following 72 h incubation in vitro. A full pharmacological profile of the compound can be found elsewhere (Quirk and Nisenbaum, 2002), and this non-specific effect was not investigated further.

In addition to monitoring neurite growth by morphometric analysis, we also measured changes in neurofilament expression. The cytoskeletal protein neurofilament has been widely used as an indicator of neurite growth and maturation (Doherty et al., 1984; Encinas et al., 2000). In agreement with our neurite growth data expression levels were robustly enhanced by AMPA potentiation and this was significantly blocked by co-incubation with the AMPA receptor antagonist CNQX. The combination of these two observations strongly suggests robust morphological plasticity following AMPA receptor potentiation.

Having tested our central hypothesis we subsequently explored the mechanisms mediating neurite growth. AMPA receptor activation can activate numerous intracellular cascades and it is unclear which is associated with the beneficial effects of AMPA receptor potentiation. Enhanced BDNF expression has been seen following AMPA potentiation in a number of systems (Lauterborn et al., 2000; Legutko et al., 2001; Mackowiak et al., 2002; Lauterborn et al., 2003). BDNF has been identified over the past decade as a modulator of a number of cellular processes including early CNS development (Ernfors et al., 1994), synaptic transmission (Patterson et al., 1996; Schratt et al., 2004) and regeneration following brain injury (Ebadi et al., 1997). It has also been hypothesised that increases in growth factors such as BDNF may lead to the beneficial effects of AMPA potentiation in a range of animal models (O'Neill et al., 2004a). Due to its role in promoting neuronal growth and survival its role in our system was investigated further.

The link between AMPA receptor activity and neurotrophins is well established (Zafra et al., 1990; Limatola, 2004). AMPA receptors can recruit a variety of second messenger molecules such as the non-receptor tyrosine kinase Lyn (Hayashi et al., 1999) FAK and PI(3)K (Millan et al., 2004). Through these they can recruit intracellular signalling cascades such as the MAP kinase pathways (Wang and Durkin, 1995; Perkinton et al., 1999; Bahr et al., 2002). AMPA receptor activation can induce the phosphorylation of CREB, thus regulating, among other genes, the expression of BDNF (Zafra et al., 1990; Finkbeiner et al., 1997; Shieh et al., 1998; Tao et al., 1998). BDNF signals primarily through the tyrosine kinase receptor Trk-B (Glass et al., 1991; Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Barbacid, 1995). Western analysis for TrK receptor expression revealed that LY404187 in the presence of AMPA can increase expression levels, suggesting an increase in neurotrophin responsiveness. To directly implicate BDNF as a mediator of neurite growth we demonstrated that BDNF antibody-sequestration attenuated the increase in neurite length following co-incubation with AMPA and LY404187. Addition of the antibody had no effect on the average length of vehicle treated cells demonstrating that the attenuated growth is due to the actions of the antibody on BDNF. Our results therefore implicated BDNF as a potential downstream effector of neurite growth following AMPA receptor potentiation.

Our results further highlight the long term neurotrophic effect of AMPA receptor potentiation and support evidence that, in addition to transient cognitive enhancement (Larson et al., 1995; Ingvar et al., 1997; Lynch, 2004), AMPA receptor potentiators can promote downstream plasticity, via the actions of neurotrophins, that may be beneficial for the treatment of chronic neurodegenerative disorders.

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