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**The impact of pharmacological modulation of the
NMDA-receptor/NO signalling pathway on
neuronal complexity *in vitro***

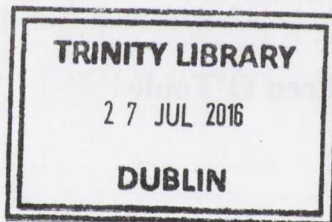
Thesis submitted to the University of Dublin, Trinity College
for the Degree of Doctor of Philosophy

By

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March 2016

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Thesis 10948

Declaration

I declare that this thesis is submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College and has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work with the exception of studies outlined in Chapter 3 (study designs: 3.2.1, 3.2.3, 3.2.4 and 3.2.7) which were conducted in conjunction with Dr. Marika Doucet.

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Summary

Neuronal atrophy and alterations in dendritic and synaptic structure are associated with multiple central nervous system (CNS) disorders ranging from acute neurological injuries to chronic neurodegenerative diseases and neuropsychiatric illnesses. Excess glutamatergic neurotransmission is increasingly associated with several of these disorders and has been suggested to contribute to the observed neuronal atrophy and synapse loss. The detrimental effects of glutamate on neurons has been particularly associated with overactivation of the glutamate N-methyl-D-aspartate receptor (NMDA-R), which couples to neuronal nitric oxide synthase (nNOS) and the nNOS adaptor protein 1 (NOS1AP) via the post-synaptic density 95 (PSD-95).

An *in vitro* model composed of rat primary cortical neurons was used in this thesis to investigate the effects of glutamatergic modulation on neurite outgrowth as measured by Sholl analysis, and synapse number as measured by quantification of the co-localised expression of the pre-synaptic marker, synaptophysin and the post-synaptic marker, post-synaptic PSD-95. Treatment of primary cortical neurons at days *in vitro* 3 (DIV 3) with glutamate (500 μ M) and NMDA (100-500 μ M) combined with glycine resulted in a decrease in neurite outgrowth. Similar effects were observed following treatment with the nitric oxide (NO) precursor, L-arginine, and NO donor, sodium nitroprusside (SNP) (100 and 300 μ M). In more mature neuronal cultures (DIV 20), NMDA (10 μ M) and L-arginine (10 μ M) decreased the co-localisation of synaptic proteins. These atrophic effects were prevented by co-treatment with the NMDA-R antagonists ketamine (10 nM) and MK-801 (10 nM), the nNOS inhibitor 1-[2-(trifluoromethyl)phenyl] imidazole (TRIM) (100 nM) and the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μ M). Small-molecule inhibitors of the PSD-95/nNOS interface, 2-((1H-benzo[d][1,2,3]triazol-5-ylamino) methyl)-4,6-dichlorophenol (IC87201) (10-100 nM) and 4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid (ZL-006) (10-100 nM), attenuated NMDA/glycine-induced decreases in neurite outgrowth and synaptic protein co-localisation. Peptide-mediated inhibition of the NOS1AP/nNOS interaction with L-TAT-GESV also counteracted NMDA/glycine-mediated neurite atrophy. A role for the p38 mitogen activated protein kinase (MAPK) pathway was also identified by showing that p38 MAPK inhibition using two commercially available inhibitors, SB203580 (1 μ M) and BIRB796 (1 μ M), in addition to the experimental peptide inhibitor, TAT-D1 (1 μ M) protected against NMDA/glycine-induced reductions in outgrowth and synaptic protein co-localisation.

The effects of astrocyte-derived factors in neuronal morphology was another main focus of this thesis, based on literature linking astrocytic dysfunctions with neuronal atrophy in several neurological disorders. Treatment of neurons with conditioned media (CM) derived from healthy

astrocytes increased neurite outgrowth and resulted in a robust increase in the co-localised expression of synaptic markers. The astrocytic toxin L-alpha aminoadipic acid (L-AAA) (0.5 and 5 mM) (24 h) reduced the oxygen consumption rate of cultured astrocytes and induced changes in the mRNA expression of glial specific markers including glial fibrillary acidic protein (GFAP), S100B and the astrocytic glutamate transporters GLAST and GLT-1, while reducing astrocyte viability after more prolonged exposure (48 h). Treatment of astrocytes with L-AAA (0.5 and 5 mM) abolished the trophic effects observed with astrocyte CM on cultured neurons. The atrophic effect of CM derived from L-AAA treated astrocytes may involve glutamatergic stimulation, as co-treatment of neurons with ketamine, as well as TRIM and IC87201 protected against L-AAA CM-induced effects on neurite outgrowth and on the co-localised expression of synaptic proteins.

These data support the hypothesis that selectively targeting the PSD-95/nNOS and NOS1AP/nNOS interaction downstream of NMDA-R promotes neurotrophic effects by preventing neuronal atrophy and potential synapse loss in response to glutamatergic stimulation. The results also highlight the importance of healthy astrocytes, and the implications of astrocytic dysfunction, in the processes governing neuronal and synapse formation. Such findings indicate that the PSD-95/nNOS and NOS1AP/nNOS protein interactions may represent attractive targets for the development of novel therapeutics aimed at restoring synaptic connections and promoting neuronal remodelling in CNS disorders associated with glutamate-induced and astrocyte-related neuronal atrophy.

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List of abbreviations

(Most frequently used abbreviations)

4E-BP1	4E binding protein 1
5-HT	5-hydroxytryptamine (serotonin)
8-Br-cGMP	8-Bromoguanosine 3',5'-cyclic monophosphate
aa	Amino acid
AA	Arachidonic acid
ABP	Actin binding protein
AC	Adenylate cyclase
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
AMPA-R	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor
AP5	D(-)-amino-5-phosphonopentanoic acid
AP5	2-amino-5-phosphonopentanoate
Arg	Arginine
BBB	Blood brain barrier
Bcl11A-L	B cell lymphoma 11A-long
BDNF	Brain-derived neurotrophic factor
BH4	Tetrahydrobiopterin
Ca ²⁺	Calcium ion
CaM	Calmodulin
CAM	Cell adhesion molecule
CaMKII	Calmodulin-dependent protein Kinase II
cAMP	Cyclic adenosine monophosphate
CAPON	Carboxy-terminal PDZ ligand of nNOS
cdc42	Cell division control protein 42
Cdk5	Cyclin dependent kinase 5
cDMEM	Complete Dulbecco's modified eagle media
cGKII	Cyclic GMP kinase II
cGMP	Cyclic guanosine monophosphate
CM	Conditioned Media
cNBM	Complete neurobasal media
CNS	Central nervous system
CPP	Cell penetrating peptide
CREB	cAMP response-element binding protein
CSPG	Chondroitin sulphate proteoglycans
ct	Carboxyl terminal

CUS	Chronic unpredictable stress
DAG	Diacylglycerol
Dexas	Dexamethasone-induced Ras protein
DGG	D-Glutamylglycine
DIV	Days <i>in vitro</i>
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
DRG	Dorsal root ganglion
EAAT	Excitatory amino acid transporter
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FGF	Fibroblast growth factor
FMN	Flavin mononucleotide
FST	Forced swim test
GABA	Gamma-amino butyric acid
GC	Guanylate cyclase
GDNF	Glial-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide-exchange factor
GFP	Green fluorescent protein
GK	Guanylate kinase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GPCR	G protein-coupled receptor
GRIP	Glutamate receptor interacting proteins
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
IB1	Islet-brain 1
IC87201	(2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl)-4,6-

	dichlorophenol)
Ig	Immunoglobulin
iGluRs	Ionotropic glutamate receptors
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP3	Inositol trisphosphate
IUPHAR	International Union of Basic and Clinical Pharmacology
JIP-1	JNK-interacting protein-1
JNK	c-Jun NH ₂ -terminal kinase
KA	Kainic acid
KATII	Kynurenine aminotransferase
kDa	Kilodalton
L-AAA	L-alpha amino adipic acid
L-Arg	L-arginine
LDH	Lactate dehydrogenase
L-NAME	N ^G -nitro-L-arginine methylester
L-NMMA	N ^G -monomethyl-L-arginine
L-NNA	N ^G -nitro-L-arginine
LTP	Long term potentiation
MAGUK	Membrane-associated guanylate kinase
MAP	microtubule associated protein
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MEK	MAPK/ERK kinase
Mg ²⁺	Magnesium ion
mGluR	Metabotropic glutamate receptor
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MPP ⁺	1-methyl-4-phenyl-pyridinium
MRI	Magnetic resonance imaging
MT	Microtubule
mTOR	Mammalian target of rapamycin
Na ⁺	Sodium ion
NADPH	Nicotinamide adenine dinucleotide phosphate
NBM	Neurobasal media
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

NCAD	N-cadherin
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NIDD	nNOS interacting DHHC domain-containing protein
NMDA	N-methyl-D-aspartate
NMDA-R	N-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS1AP	Nitric oxide synthase adaptor protein 1
NOS1AP-L	Nitric oxide synthase adaptor protein 1 long
NOS1AP-S	Nitric oxide synthase adaptor protein 1short
NPC	Neural progenitor cell
NT	Neurotrophin
ODQ	1H-[1,2,4]oxadiazolo[4,3,-a] quinoxalin-1-one
OGD	Oxygen and glucose deprivation
p70S6K	p70 ribosomal S6 kinase
PARP	Poly (ADP-ribose) polymerase
PCP	Phencyclidine
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase 1
PDZ	PSD-95/discs large/ZO-1 homologous
PFC	Prefrontal cortex
PFK-M	Phosphofructokinase isoform M
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PI3-kinase	Phosphatidylinositol 3-kinase
PICK 1	Protein interacting with C kinase 1
PIP2	Phosphatidylinositol (4,5)-diphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	Phopholipase C
PSD	Post synaptic density

PSD-95	Post synaptic density 95 kDa
PTB	Phosphotyrosine binding domain
QA	Quisqualic acid
RGC	Retinal ganglion cell
RNA	Ribonucleic acid
ROCK	RhoA/Rho kinase
ROS	Reactive oxygen species
S100B	S100 calcium-binding protein B
SAP	Synaptic associated protein
SAPK	Stress activated protein kinase
SERT	Serotonin (5-HT) transporter
sGC	Soluble guanylate cyclase
SH3	Src homology 3
SIN-1	3-morpholiniosydnonimine
siRNA	Small interfering RNA
SNAP	S-Nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside
Tat	Trans-activating transcriptional activator
TBI	Traumatic brain injury
TNF	Transforming growth factor
TRIM	1-[2-(trifluoromethyl)phenyl] imidazole
Trk	Tyrosine receptor kinase
TSP	Thrombospondin
TST	Tail suspension test
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling
VEGF	Vascular endothelial growth factor
Vglut	Vesicular glutamate transporter
ZL006	(4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid)

Chapter 1: General Introduction

1.1 Neuronal atrophy and synapse loss in central nervous system (CNS) disorders

The formation and maintenance of correct neuronal circuits and synaptic connections in the central nervous system (CNS) is crucial to the functional integrity of the brain. During CNS development, axons and dendrites (collectively referred to as neurites) grow in a highly coordinated manner to cover their target receptive field and become optimally positioned to receive and integrate signals from neighbouring neurons. Impairments in the process of neurite patterning and synapse formation during brain development are associated with several neurodevelopmental disorders including schizophrenia and autism (Geschwind and Levitt, 2007, Mei and Xiong, 2008, Freudenberg et al., 2015). Moreover, the mature CNS - which was originally assumed to have a limited capacity for neuronal modifications once it was fully developed - is now recognised as being highly plastic and responsive to environmental and physiological cues which can lead to alterations in dendritic arborisation and synaptic structure throughout life (Gould, 2007, Ho et al., 2011). These neuroplastic changes are believed to underpin the processes of learning and memory and include the phenomena of adult hippocampal neurogenesis which involves the integration of new neural precursor cells (NPCs) into existing neuronal networks, as well as synaptic plasticity which involves activity-dependent changes in the strength of synapses (Martin et al., 2000, Zhao et al., 2008, Deng et al., 2010). In addition, alterations in the morphology and connectivity of existing neurons and synapses in the adult brain are increasingly considered a hallmark of brain injury, and can occur as either a cause or a consequence of disease pathology (Fiala et al., 2002).

Neuronal atrophy, aberrant dendrite morphology and synaptic abnormalities are prominent pathological features of many CNS disorders ranging from ischaemia to neuropsychiatric disorders as well as neurodegenerative disorders such as Alzheimer's and Parkinson's disease. For instance, loss of dendrite spine and arbour stability is known to occur within and adjacent to the infarct area following stroke (Koleske, 2013). These atrophic events represent some of the earliest changes in the ischaemic brain and precede subsequent cell death (Monnerie et al., 2003). Targeting these early morphological neuronal deficits may therefore represent a strategy to prevent subsequent cell death and restore neuronal function following ischaemic damage. Compelling evidence from both animal and human studies indicate that stress and depression are associated with neuronal atrophy, dendrite shrinkage and decreased synapse density in several brain regions including the cerebral cortex and hippocampus (Duman et al., 2000, Duman, 2002, Banasr et al., 2011). Animals exposed to repeated stress exhibit dendrite retraction and shortening, as well as alterations in spine and synapse number in brain regions such as the

hippocampus and prefrontal cortex (PFC) (McEwen and Morrison, 2013). Neuronal atrophy has long been recognised as a major hallmark of neurodegenerative diseases such as Alzheimer's disease (Swaab et al., 1994). Significant neuronal loss and synaptic structural alterations in addition to the accumulation of amyloid plaques and tau protein neurofibrillary tangles characterise this illness, and the loss of synaptic connections are thought to play a major role in contributing to the cognitive decline associated with Alzheimer's disease. Moreover, prominent neuronal and synaptic deficits also feature in disorders such as autism, Down syndrome and Fragile X syndrome (see **Table 1.1** for reference to detailed relevant reviews). Although the mechanisms underlying atrophy vary depending on the illness, it is likely that deficits in neuronal connectivity have a profound effect on brain function and contribute to the pathology of many of these illnesses. In this regard, mechanisms aimed at encouraging neurite outgrowth and preventing synapse loss could provide a means to restore neuronal functional connectivity in several disease states, and may represent a strategy for cognitive improvement and therapeutic relief in these illnesses.

Table 1.1: Neurological disorders commonly associated with neuronal atrophy, spine loss and synaptic alterations

	Pathology:	Brain region/s (if relevant):	Reviewed in:
Neurodevelopmental/genetic disorders			
Autism	Increased spine density, increased synapse number	Cortex, Hippocampus	(Bourgeron, 2009, Bakos et al., 2015)
Down Syndrome	Decreased dendrite length and branching , reduction in spine and synapse density	Hippocampus	(Fiala et al., 2002, Kurt et al., 2004)
Fetal Alcohol Syndrome	Spine and synapse loss, long spines, reduced spine density,	Cortex	(Fiala et al., 2002, Granato and De Giorgio, 2014)
Fragile X Syndrome	Increased number of spines and synapses, long spine size	Cortex, Hippocampus	(Irwin et al., 2000, He and Portera-Cailliau, 2013)
Rett's Syndrome	Decreased dendrite branching	Frontal cortex, Motor cortex	(Banerjee et al., 2012, Kulkarni and Firestein, 2012)
Schizophrenia	Reduced dendritic arbor, decreased spine density, smaller spines	Cortex, Hippocampus	(Eastwood and Harrison, 2001, Glantz et al., 2006)
Other CNS disorders			
Alcohol and drug abuse	Neuronal loss	Hippocampus, Cerebellum	(Fiala et al., 2002, Dlugos, 2015)
Epilepsy	Spine loss, varicosity formation	Hippocampus	(Fiala et al., 2002, Casanova et al., 2012)
Stress, Anxiety, Depression	Decreased dendrites, spine loss	Hippocampus, PFC	(Popoli et al., 2012, McEwen and Morrison, 2013, Duman, 2014)
Ischaemia	Spine loss, varicosity formation, dendrite atrophy	Infarct area	(Moskowitz et al., 2010, Koleske, 2013)
Traumatic brain injury	Anterograde and retrograde axonal degeneration		(Fiala et al., 2002, Blennow et al., 2012)
Neurodegenerative disorders			
Alzheimer's	Neuronal loss, reduced dendrite arbor, spine/synapse loss	Hippocampus, neocortex	(He et al., 2009, Palop and Mucke, 2010)
Parkinson's	Neuronal loss, spine/synapse loss	Substantia nigra	(Fiala et al., 2002, Day et al., 2006)

1.2 The process of neurite outgrowth

The processes underlying the formation of the elaborate dendritic trees and axonal patterns arising from a neuron’s soma have been the subject of numerous extensive studies and reviews (Scott and Luo, 2001, da Silva and Dotti, 2002, Goldberg, 2003). The initial stages of neurite formation and outgrowth have been characterised most comprehensively in rat embryonic hippocampal neurons in culture and can be classified into four distinct steps (**Figure 1.1**). Firstly, the original neuronal sphere is broken down and an initial bud forms which becomes the first neurite. Secondly, new primitive neurites (termed minor processes) begin to develop and these neurites become polarised, meaning that the axon and dendrites become morphologically distinct. Neurites then begin to elongate in defined directions and start to form branches. Lastly, neurites reach their defined targets, develop spines (the sites of the majority of synapses), and can therefore form pre- and post-synaptic connections with neighbouring neurons, allowing for synaptic transmission. The development of a neuron is not complete after these initial stages of development however.

Instead, neurites and synapses undergo extensive pruning during development to eliminate unnecessary or incorrect connections, as well as being subject to changes in spine and synapse number and efficacy in response to environmental and physiological responses throughout life. The molecular processes and cytoskeletal changes underlying initial neurite patterning during development have been reported to be similar to those that control new spine formation and plasticity in adulthood (Luo, 2002). Therefore, elucidation of the mechanisms and factors influencing neurite outgrowth during development may be of relevance not only to understanding neurodevelopmental disorders associated with altered neuronal morphology, but also for promoting neuronal remodelling in the adult brain. This is of interest for the promotion of axonal regeneration following injury (Shortland et al., 2006), for the functional integration of neuronal precursor cells (NPCs) into existing neuronal networks (Deng et al., 2010) , and also for the prevention or reversal of dendritic atrophy and spine loss observed in neurodegenerative diseases, ischaemia and neuropsychiatric disorders (Banasr et al., 2011, Duman, 2014).

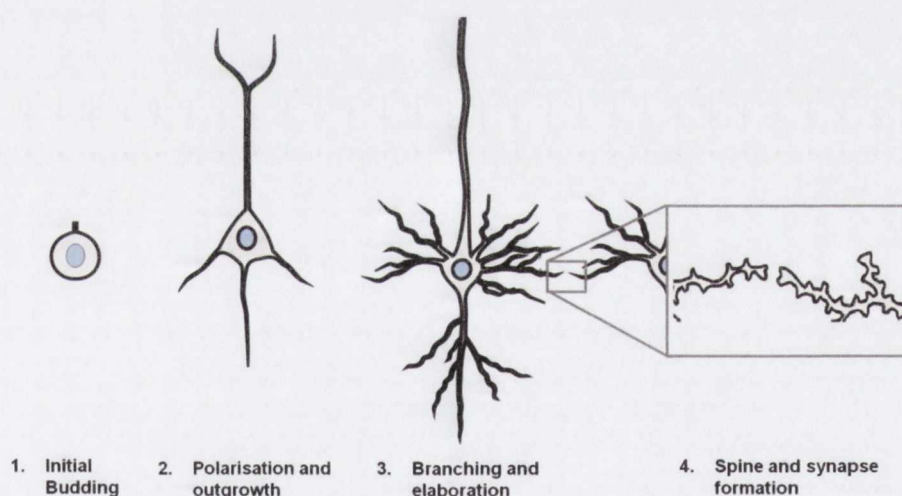


Figure 1.1: Stages of Neurite Outgrowth. The process of neurite outgrowth can be classified into four steps with occur in a dynamic and overlapping manner; **1)** Initial budding; **2)** Polarisation and elongation of axons and dendrites; **3)** Branching and elaboration; **4)** Spine and synapse formation.

1.2.1 The role of the cytoskeleton and growth cone in neurite outgrowth

Neurons are guided through their environment by their specialised and highly responsive terminals called growth cones, and the process of neurite outgrowth is fundamentally based on the coordinated changes occurring in the dynamic cytoskeleton of this region of the neuron [for

detailed reviews see Luo (2002) , Goldberg (2003) and Lowery and Van Vactor (2009)]. Growth cones can be divided into three main compartments based on the localisation of their underlying cytoskeletal constituents which are composed of filamentous actin (F-actin) and microtubule (MT) polymers. Actin-based finger-like filopodia and sheet-like lamellipodia comprise the highly dynamic peripheral (P) domain of the growth cone. Microtubule polymers along with other organelles and vesicles comprise the more stable central (C) domain. The P and C domains are flanked by a transitional (T) zone, which consists of arcs of actin and myosin II motor proteins (Luo, 2002, Lowery and Van Vactor, 2009) (**Figure 1.2**). This distribution of cytoskeletal constituents allows a neurite to grow according to the three stages of protrusion, engorgement and consolidation. Protrusion relates to the extension and polymerisation of F-actin-based filopodia and lamellipodia, accompanied by membrane extension at the edge of the growth cone. Engorgement involves the invasion of microtubules into the peripheral region, bringing with them new organelles and vesicles. Finally, consolidation refers to the stabilisation and depolymerisation of F-actin in the former growth cone, allowing the membrane to shrink around the underlying microtubules and converting this region into a new area of the cylindrical axon shaft (Lowery and Van Vactor, 2009, Dent et al., 2011).

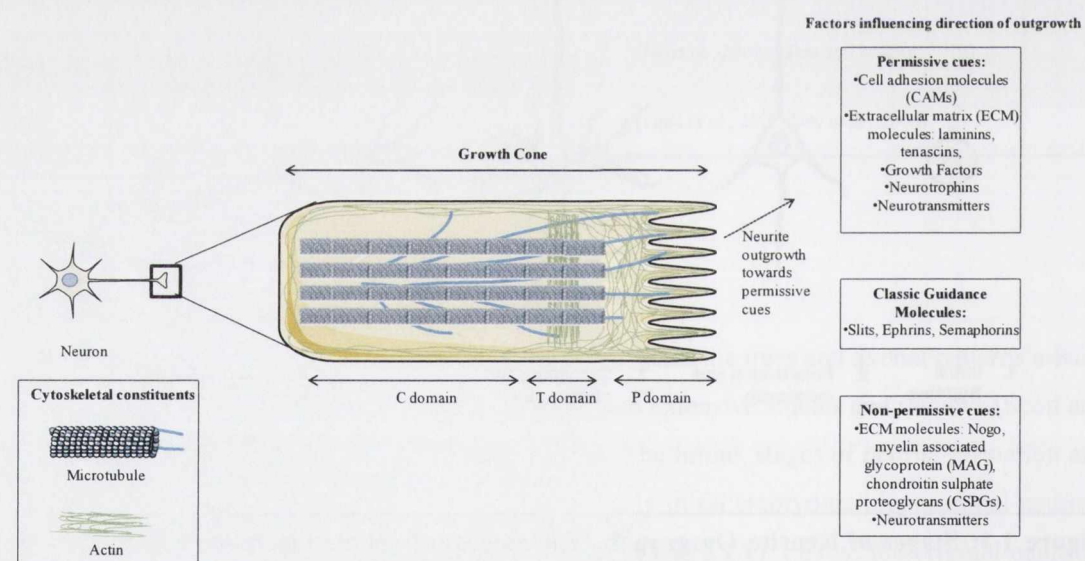


Figure 1.2: The neuronal growth cone and neurite guidance. Neurons are guided through the extracellular environment through their highly responsive growth cones. Actin based finger-like filopodia and sheet-like lamellipodia comprise the highly dynamic peripheral (P) domain of the growth cone while the more stable central (C) domain is rich in microtubules. These regions are flanked by the transitional (T) domain which is composed of actin arcs and myosin II motor proteins. The direction and rate of neurite outgrowth is determined by the presence of both substrate-bound and diffuse gradients of molecules in the extracellular environment which can act as either permissive (attractive) or non permissive (repellent) factors.

1.2.2 Guidance Cues for neuronal pathfinding and outgrowth

A wide array of signalling factors ranging from substrate-bound extracellular matrix (ECM) molecules to diffusible gradients of neurotransmitters and trophic factors have been shown to play important roles in influencing neurite outgrowth and regulating neuronal morphology. Examples include ECM molecules such as permissive laminins and tenascins (Kamiguchi and Lemmon, 2000, Li et al., 2008) and repulsive proteoglycans (Snow et al., 1990, Snow and Beller, 2014), cell adhesion molecules (CAMs) (Williams et al., 1994, Ranscht, 2000, Rigato et al., 2002, Kiryushko et al., 2004, Hansen et al., 2008), ‘classic’ guidance molecules including slits, ephrins and semaphorins, as well as various neurotrophins and neurotransmitters (Gallo and Letourneau, 2000). These extracellular cues, and the signalling cascades initiated by them, ultimately converge on neuronal proteins interacting directly with the actin and microtubule cytoskeleton, thus promoting or inhibiting outgrowth (Dent et al., 2011). Such proteins include Rho GTPases, which are small (~21kDa) G-proteins from the Ras superfamily and are regarded as direct mediators of the cytoskeleton. Rho GTPases include members such as RhoA, RAC1 and cell division control protein 42 (Cdc42), and act by linking extracellular cues such as nitric oxide (NO) (Sunico et al., 2010) and kinases (Khodosevich and Monyer, 2010) to actin dynamics, and therefore directly mediate cytoskeletal responses to various extracellular stimuli (Hall and Lalli, 2010, Polleux and Snider, 2010). Although it was originally thought that these extracellular signals act as either permissive (attractive) or non-permissive factors (repellent), it is now believed that the response taken by developing growth cones to these cues can vary depending on the type of neuron, the receptors present on the growth cone, and the asymmetric subcellular distribution of intracellular signalling proteins needed for a cytoskeletal response (Kiryushko et al., 2004). For example some chemotrophic cues can act as both an attractive and a repulsive source to the same neuron depending on subcellular localisation of intracellular signalling components (Polleux et al., 2000). In addition, external cues influencing outgrowth can also interact with each other creating a dynamic web of signalling responses in the neuron which ultimately shape its navigation pattern and final morphology (Kiryushko et al., 2004) (see **Figure 1.2** for factors impacting growth cone guidance).

1.3 The process of synapse formation

Synapses are specialised junctions occurring between neurons which allow for interneuronal communication via neurotransmitters and form the basis of most information transfer in the CNS (McAllister, 2007). Synaptogenesis occurs when pre-synaptic axons approach correct targets and establish connections with post-synaptic dendritic arbours. Synapses can occur at the growth

cones of developing neurites as well as at multiple sites along the length of axonal and dendrite shafts. Excitatory synapses are particularly concentrated at dendritic spines, which refer to neuronal protrusions that act to compartmentalise calcium influx and localise neurotransmitter receptors and synaptic proteins that are needed for cellular signalling pathways (Hering and Sheng, 2001, Fiala et al., 2002, Nimchinsky et al., 2002). The process of synaptogenesis begins with the formation of an unspecialised contact between an axon terminal and a developing dendrite. Thousands of synaptic vesicles begin to accumulate at the pre-synaptic terminal and neurotransmitter release is triggered by the fusion of these vesicles to the pre-synaptic membrane at an area referred to as the active zone. Neurotransmitters cross the synaptic cleft before reaching the post-synaptic membrane which becomes differentiated through the coordinated accumulation of specialised membrane protein complexes - such as the post-synaptic density 95 (PSD-95) - and post-synaptic neurotransmitter receptor accumulation (Cline, 2001, Cohen-Cory, 2002, McAllister, 2007). The accumulation of receptors at the post-synaptic site is regarded as a key feature in the specialisation of synapses. Of particular interest, at glutamatergic synapses, the expression of glutamate N-methyl-D-aspartate (NMDA) receptors (NMDA-R) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPA-R) are developmentally regulated such that NMDA-R expression precedes that of AMPA-R expression, and NMDA-R activation is known to modulate AMPA-R membrane translocation to the membrane. The interplay between NMDA and AMPA activity at developing synapses is known to play a key role in the processes underlying synaptic plasticity and long term potentiation (Malinow and Malenka, 2002, Derkach et al., 2007).

The processes of synapse formation and neurite development are tightly linked, and research suggests that synapse formation is concurrent with, and facilitates, dendrite development (Niell et al., 2004). For instance, long term imaging studies carried out on non-spiny dendritic arbours in zebrafish have demonstrated that the formation of the majority of synapses occurs initially on newly extended dendritic filopodia, and these synapses act to stabilise the developing filopodia. These results have led to the 'synaptotropic hypothesis' of dendrite growth, which posits that synapse formation acts to strengthen filopodia and hence plays a key role in determining the final morphology of dendritic trees (Niell et al., 2004, Meyer and Smith, 2006). Like neurite outgrowth therefore, the precise formation of synapses on dendritic arbours is crucial to the correct functioning of the CNS and underlies cognition, perception, learning and memory (McAllister, 2007). During neurodevelopment, an overabundance of synapse formation occurs, and incorrect synaptic connections are eliminated through the important process of synaptic pruning. This is exemplified by illnesses in which improper synapse development is highly implicated including neurodevelopmental disorders such as autism and Fragile X syndrome (Scheiffele, 2003, McAllister, 2007). Autism for instance, is increasingly regarded as a disconnectivity syndrome

featuring an excess in synaptic connections resulting from a deficit in the normal process of synaptic pruning (Geschwind and Levitt, 2007), while some evidence suggests the opposite (i.e. an excess of synaptic pruning) may occur in the PFC and contribute to schizophrenia pathology (Keshavan et al., 1994). In the adult brain, regulation of synapse number is also tightly controlled, and it has also been reported that microglia, which become activated in response to CNS damage and inflammation, can initiate the process of 'synaptic stripping', which refers to the removal of dysfunctional synapses in many disease states (Kettenmann et al., 2013). In addition, synapse loss, rather than cell death, is increasingly regarded as the predominant neuronal pathology responsible for cognitive decline in Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Sunico et al., 2010). Understanding the mechanisms underlying synapse and spine loss in these disorders is therefore of key interest for the development of therapeutic strategies aimed at counteracting synaptic alterations.

1.4 Glutamate neurotransmission

Glutamate is the main excitatory neurotransmitter in the CNS and is crucial for normal brain function. Glutamate is regarded as a key neurotransmitter for regulating neurogenesis, synaptogenesis, neurite outgrowth and apoptosis in both the developing and adult CNS (Mattson, 2008). Glutamate neurotransmission is of particular importance for modulating synaptic plasticity and for the mechanisms underlying higher order processes such as learning and memory. Furthermore, glutamate is the precursor for gamma-aminobutyric acid (GABA) synthesis - the major inhibitory neurotransmitter in the brain. Glutamate is widely and relatively uniformly distributed in the CNS and is synthesised from glucose via the Krebs cycle, or recycled by astrocytes via the glutamate/glutamine shuttle (Willard and Koochekpour, 2013). In the pre-synaptic terminal, glutamate is packaged and stored into synaptic vesicles by vesicular glutamate transporters (VGLut) and is released into the synapse through Ca^{2+} dependent exocytosis. Once released, it mediates its actions through its post-synaptic cell surface receptors and is then rapidly removed from the synapse through re-uptake into the pre-synaptic terminal via class II and III metabotropic glutamate receptors (mGluRs), and also through high affinity excitatory amino acid transporters (EAATs) which are located on the pre-synaptic neuron and also on the surface of astrocytes. Astrocytes are crucial to glutamate metabolism - they prevent the excessive and potentially damaging build up of glutamate in the synapse, and they also possess the enzyme glutamine synthetase which converts glutamate to glutamine. Astrocytic glutamine can then be transported back to neurons where a glutaminase enzyme converts it to glutamate - thereby allowing glutamate to be recycled and completing the glutamate/glutamine cycle (**Figure 1.3**).

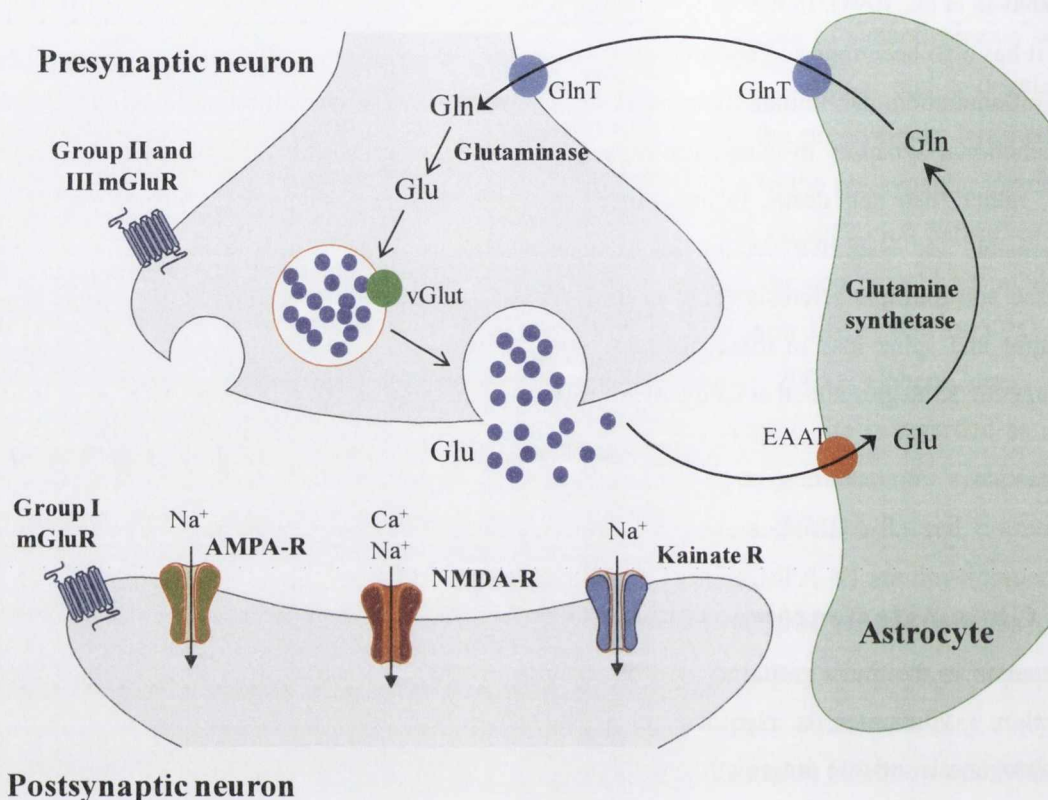


Figure 1.3: The glutamatergic synapse. Glutamate (Glu) is packaged into vesicles in the pre-synaptic neuron by the vesicular glutamate transporters (vGluT) and is released into the synaptic cleft where it activates post-synaptic ionotropic glutamate receptors (NMDA-R, AMPA-R and kainate-R) and metabotropic glutamate receptors (mGlu-R). mGlu-Rs are also present on the pre-synaptic neuron which are involved in regulating glutamate release. Glutamate is removed from the synapse through excitatory amino acid transporters (EAATs) which are located on neighbouring astrocytes and is converted to glutamine (Gln) by the enzyme glutamine synthetase. Glutamine is then shuttled back to neurons where it can be converted to glutamate by glutaminase.

1.5 Glutamate receptors

Glutamate mediates its excitatory effects via activation of neuronal cell surface receptors comprising of the metabotropic glutamate receptors (mGluRs) and the ionotropic glutamate receptors (iGluRs) (Dingledine et al., 1999). mGluRs are G-protein coupled receptors which contain the classical seven transmembrane domain structure, while iGluRs are ligand-gated ion channels that allow cation influx upon glutamate binding. Both mGluRs and iGluRs can be

further classified based on their sequence homology, pharmacology and electrophysiological properties (**Figure 1.4**).

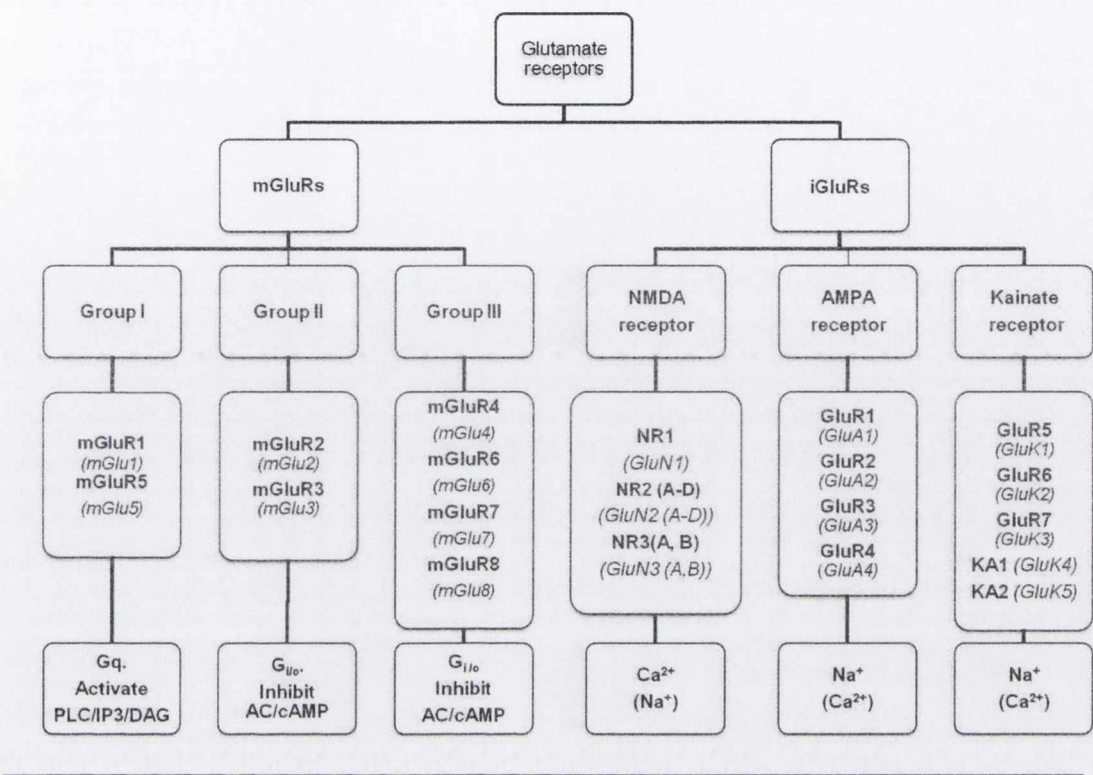


Figure 1.4: Overview of the family of glutamate receptors. Glutamate receptors comprise of metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). mGluRs are G-protein coupled receptors (GPCRs) which mediate their effects through G proteins (excitatory Gq or inhibitory Gi/Go), and intracellular signalling cascades including activation of the phospholipase C/inositol 1,4,5-triphosphate/diacylglycerol (PLC/IP3/DAG) pathway or inhibition of adenylate cyclase (AC). iGluRs are ligand gated receptors that allow cation [calcium (Ca²⁺) and/or sodium (Na⁺)] influx upon glutamate binding. Receptor subtypes are named according to frequently used names (bold font), while the corresponding International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature is included in italics underneath.

1.5.1 Metabotropic glutamate receptors (mGluRs)

mGluRs are widely distributed in the CNS and modulate cell excitability via second messenger signalling pathways. mGluRs contain a large extracellular N-terminal domain which glutamate binds with (referred to as the venus flytrap domain due to its unique shape), a seven transmembrane domain and an intracellular c-terminal domain which can undergo alternative splicing, allowing for different interactions with scaffolding proteins (Willard & Koochekpour 2013). There are eight types of metabotropic glutamate receptor subtypes (mGluR₁₋₈) which are categorised into group I, II and III subfamilies. Group I (mGluR1 and mGluR5) are predominantly found at post-synaptic sites. They are coupled to Gq proteins and act to enhance glutamate excitation by initiating signalling cascades involving the activation of phospholipase

C/inositol 1,4,5-triphosphate/diacylglycerol (PLC/IP3/DAG). Group II mGluRs (mGluR2 and mGluR3) and group III mGluRs (mGluR4, 6, 7, and 8) are primarily located pre-synaptically and are coupled to the inhibitory G-proteins, G_i/G_o . Their activation results in the inhibition of adenylate cyclase (AC) activity and reduced cyclic adenosine monophosphate (cAMP) levels, and their functions include inhibiting glutamate release and reducing neuronal excitability (Willard and Koochekpour, 2013).

1.5.2 Ionotropic glutamate receptors (iGluRs)

iGluRs are widely distributed in the CNS and are divided into three main types which are named after the selective agonists that were originally found to activate them: the α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPA) receptor, the kainate receptor and the N-methyl-D-aspartate (NMDA-R) receptor. Functional iGluRs are tetramers composed of homo-oligomeric or hetero-oligomeric assemblies of distinct subunits. The variety in possible subtype conformations accounts for a heterogeneous mix of iGluRs in the CNS, with each displaying different response characteristics (Scannevin and Huganir, 2000). Each iGluR subunit consists of an extracellular N terminus, intracellular c-terminus (ct) and three transmembrane domains (M1, M3 and M4) in addition to a cytoplasm-facing re-entrant loop (M2) that does not cross the membrane (Dingledine et al., 1999). AMPA receptors (AMPA-Rs) and kainate receptors mediate fast electrophysiological responses to glutamate whereas NMDA receptors (NMDA-Rs) mediate slower responses.

1.5.2.1 AMPA receptor (AMPA-R)

AMPA-Rs are distributed throughout the CNS and are composed of a family of four subunits called GluR1-4 which assemble in homomeric or heteromeric structure to form functional tetramers (Kew and Kemp, 2005). AMPA-R activation results in an influx of mostly Na^+ and some Ca^{2+} . The degree of Ca^{2+} permeability is dependent on the subunit composition, with AMPA-Rs containing the GluR2 subunit being relatively impermeable to Ca^{2+} . The reason for this is due to an arginine at a critical site in the M2 domain of GluR2 instead of a glutamine which is found in the corresponding site on the other subunits (Dingledine et al., 1999). Another level of complexity in AMPA-Rs composition arises from the fact that all four subunits can exist in either of two alternatively spliced forms – so called ‘Flip’ and ‘Flop’ - which can influence AMPA-R desensitisation and allosteric modulation properties (Kew and Kemp, 2005). Similar to mGluRs, the ct of AMPA-R subunits play an important role in interacting with scaffolding proteins which help to link signalling molecules and neuronal proteins to the receptor, therefore aiding in signal transduction pathways. For example, the ct of GluR2 and GluR3 AMPA-R subunits bind to the

glutamate receptor interacting proteins 1 and 2 (GRIP1, GRIP2) and protein interacting with C kinase 1 (PICK 1). In addition, the GluR1 subunit is known to bind to synaptic associated protein 97 (SAP 97). The binding of these molecules to AMPA-R is thought to play a key role in AMPA-R membrane trafficking and in signal transduction by interacting with downstream signalling molecules. For example PICK-1 interacts with protein kinase c (PKC) leading to subsequent downstream phosphorylation cascades (Scannevin and Huganir, 2000).

1.5.2.2 Kainate receptor

Kainate receptor subtypes show high expression levels in the cerebellum, hippocampus, spinal cord and neocortex (Bleakman and Lodge, 1998). Kainate receptors are composed of two subunit families – GluR5-7 and KA-1 and -2. Like AMPA-Rs, kainate receptors consist of a tetramer of homomeric or heteromeric assemblies of these subunits. KA1 and KA2 subunits do not form functional homomeric receptors but instead have high affinity kainate binding sites and can combine in heteromeric assemblies with GluR5-7 to form functional receptors (Bleakman and Lodge, 1998, Kew and Kemp, 2005). Kainate receptor subunits can also undergo alternative splicing and editing - like AMPA-R subunits - thus increasing the level of heterogeneity observed with these receptors (Dingledine et al., 1999). Progress in understanding the function of kainate receptors is less advanced than that of the other GluRs which is attributed to the lack of specific agonists and antagonists (Jane et al., 2009). However, kainate receptors have been shown to play a role in regulating excitatory transmission at mossy fibre synapses in the CA3 region of the hippocampus where they regulate both pre- and post-synaptic transmission. Kainate receptors also modulate the strength of synaptic transmission and regulate the release of GABA and glutamate (Kew and Kemp, 2005).

1.5.2.3 NMDA receptor (NMDA-R)

The NMDA-R family is composed of seven subunits, namely NR1, NR2 (A-D) and NR3A and B with eight splice variants existing for the NR1 subunit (Dingledine et al., 1999, Kew and Kemp, 2005). The different receptor subunits are known to be spatio-temporally distinct. For example, NR2A and NR2B are the predominant subtypes in the rodent and human forebrain, while the NR2D subunit appears to be restricted to early development and is expressed in the diencephalon and midbrain (Kew and Kemp, 2005). The functional NMDA-R forms a tetrameric receptor complex, typically consisting of two NR1 and two NR2 subunits. The NMDA-R exhibits a number of special features which ultimately endow it with its unique pharmacological properties. For instance, NMDA-R, unlike AMPA-R, is highly permeable to Ca^{2+} which enters the cell upon activation of its central ion channel. Channel opening requires glutamate binding to the NR2

subunit, and also requires the co-agonist, glycine, binding to the NR1 subunit. Under normal resting membrane conditions (-65mV), the NMDA-R ion channel is blocked by magnesium ions (Mg^{2+}) which keep the receptor inactive regardless of glutamate binding. However, AMPA-R-mediated depolarisation of the post-synaptic cell results in Mg^{2+} release from the NMDA-R ion channel, thus allowing for activation of NMDA-R and subsequent Ca^{2+} influx. The Mg^{2+} block under resting conditions accounts for the slower transmission mediated by NMDA-R compared to AMPA-R and kainate receptors (Kew and Kemp, 2005).

1.6 Nitric oxide (NO) and nitric oxide synthase (NOS)

Nitric oxide (NO) is a gaseous transmitter which has key physiological roles in cardiovascular, gastrointestinal, genitourinary, respiratory and nervous systems (Vallance and Leiper, 2002). In the CNS, it has important physiological roles in neurotransmission, synaptic plasticity, pain perception and in modulating certain behaviours such as aggression (Dhir and Kulkarni, 2011). NO is synthesised by a family of enzymes, the nitric oxide synthases (NOS), which have an almost ubiquitous distribution in the body. There are three subtypes of NOS with a sequence homology of 50-60% named according to the cell type and/or conditions in which they were first described: endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1), and inducible NOS (iNOS or NOS2). Mitochondria are also known to contain a NOS enzyme - termed mitochondrial (mt) NOS 'mtNOS'- which is thought to be a nNOS isoform with two post-translational modifications (Elfering et al., 2002). All NOS isoforms catalyse the oxidation of L-arginine to citrulline and NO in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 . nNOS and eNOS are constitutively expressed and exist as an inactive monomer requiring dimerisation for activation. In contrast, iNOS - a Ca^{2+} /calmodulin (CaM) independent enzyme - requires *de novo* synthesis and is expressed following inflammatory or immunological stimulation in microglia, astrocytes, macrophages and other cells, generating significant quantities of NO that can persist for hours or days (Vallance and Leiper, 2002, Pacher et al., 2007). All NOS isoforms contains high affinity binding sites for L-arginine (Arg), haem and tetrahydrobiopterin (BH_4) which comprise the oxygenase domain of the enzyme. In addition, cofactor binding sites are present including flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH) which comprises the reductase domain. The dimerisation of NOS creates high affinity binding sites for Arg/Haem/ BH_4 . The NADPH, FAD and FMN cofactor binding sites allow electrons donated by NADPH to transfer from the reductase domain to the oxygenase domain via FAD, FMN and CaM. The CaM binding site regulates this electron flow and in the presence of Ca^{2+} , CaM associates to Ca^{2+} creating a Ca^{2+} /CaM complex, resulting in electron flow and oxidation of L-arginine to form L-citrulline and NO (Figure 1.5).

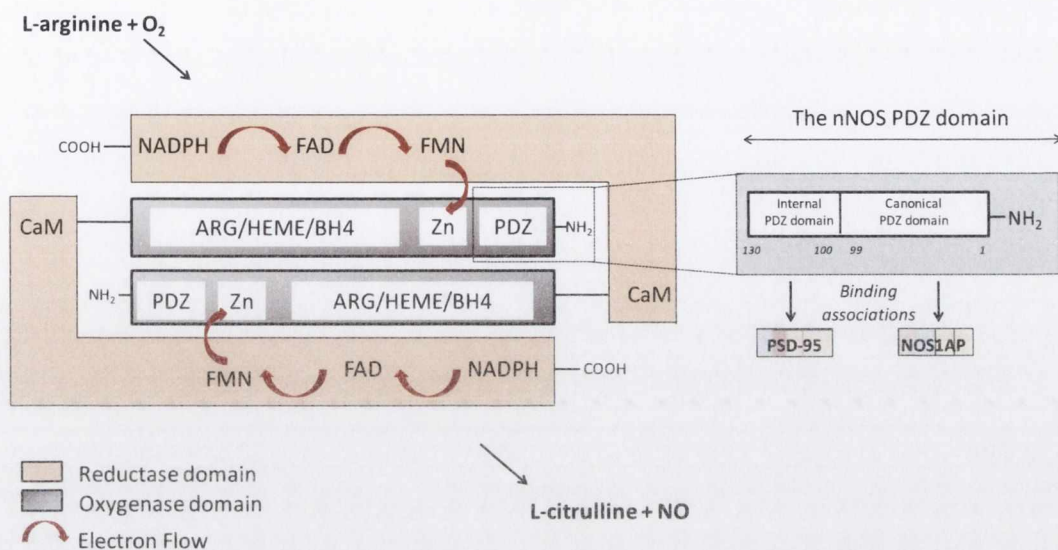


Figure 1.5: Neuronal nitric oxide synthase (nNOS) dimer. nNOS is active in dimer form and consists of two identical monomer subunits. Each monomer has a reductase domain (C-terminal) and an oxygenase domain (N terminal) which are separated by a calmodulin (CaM) binding motif. The reductase domain contains flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN) binding sites while the oxygenase domain contains the binding sites for the substrate L-arginine (ARG) as well as binding sites for heme, tetrahydrobiopterin (BH₄) and zinc (Zn) which facilitates dimerisation. Electrons donated by nicotinamide adenine dinucleotide phosphate (NADPH) transfer from the reductase domain of one monomer to the oxygenase domain of the second monomer via FAD, FMN and CaM. **Inset:** The extended PDZ domain of nNOS is located at the N-terminus (NH₂) and comprises of a canonical PDZ domain at amino acid residues 1-99 which binds to several proteins including NOS1AP, and also contains an internal PDZ domain (so called β -finger) at residues 100-130 which is believed to bind to the post-synaptic density-95 (PSD-95).

1.7 NO signalling

NO has a half life of 30 to 60 seconds and is considered an atypical neurotransmitter with several unique properties. For example, it is not stored in synaptic vesicles or released via exocytosis, but instead is produced upon demand by nNOS and diffuses freely across cell membranes where it can interact directly with intracellular signalling components. Soluble guanylate cyclase (sGC) is regarded as the most sensitive receptor for NO (Bellamy and Garthwaite, 2002). NO activates sGC by binding to the heme site on the protein and inducing a conformational change. sGC activation triggers the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP acts as a second messenger, and its production results in activation of protein kinases including PKG I and II, phosphodiesterases (PDE III) and cGMP-dependent kinase II (cGKII). This pathway has been shown to play a role in synaptic plasticity, and research

has demonstrated that the activation of cGKII by cGMP increases membrane insertion of GluR1 containing AMPA-Rs at extrasynaptic sites, and may therefore enhance extrasynaptic AMPA-R transmission. The increase in AMPA-Rs has been reported to occur through cGKII binding to the ct domain of GluR1 and phosphorylating GluR1 at Ser845 – a property which was found to increase GluR1 membrane insertion. Blocking cGKII activity prevents the surface increase of GluR1 and has the functional consequence of reducing long term potentiation (LTP) in hippocampal slices (Serulle et al., 2007). In addition, cGMP-mediated activation of PKG has been shown to activate RhoA/Rho kinase (ROCK) signalling and results in actin depolymerisation and subsequent synapse elimination in motoneurons (Sunico et al., 2010). This indicates that signalling downstream of nNOS-dependent NO production plays a role in neuronal and synaptic morphology.

NO can also mediate effects independently of sGC activation and cGMP production, and can interact directly or indirectly with metals, reduced thiols, molecular oxygen and other reactive oxygen species. For instance, NO associates with superoxide anion (O_2^-) which leads to the formation of peroxynitrite ($ONOO^-$), an oxidant that can modify proteins and lipids by direct oxidative reactions or via indirect, radical-mediated mechanisms (Vallance and Leiper, 2002, Pacher et al., 2007). Furthermore, NO can interact with cyclooxygenase and heme oxygenase I, proteins involved in inflammatory and stress response systems, as well as regulating the Akt kinase signalling pathway and the transcription factor cyclic-AMP-responsive-element-binding protein (CREB) (Calabrese et al., 2007). NO can also directly modify proteins through S-nitrosylation (also called S-nitrosation), a process which is increasingly regarded as an important mechanism by which NO exerts effects on processes such as synaptic plasticity, synaptogenesis and neurogenesis (Hardingham et al., 2013, Santos et al., 2014). S-nitrosylation refers to a post-translational modification of thiol groups (mainly cysteines) of various target proteins, leading to functional modifications. For example, NO mediates S-nitrosylation of the GluR1 AMPA-R subunits, which has been reported to increase AMPA-R membrane insertion, thereby facilitating LTP and synaptic plasticity (Selvakumar et al., 2013). In addition, dexamethasone-induced Ras protein 1 (Dexas1) is known to be S-nitrosylated by NO, a process which is facilitated by the scaffolding properties of nNOS adaptor protein 1 (NOS1AP) (Fang et al., 2000) (**Figure 1.6**).

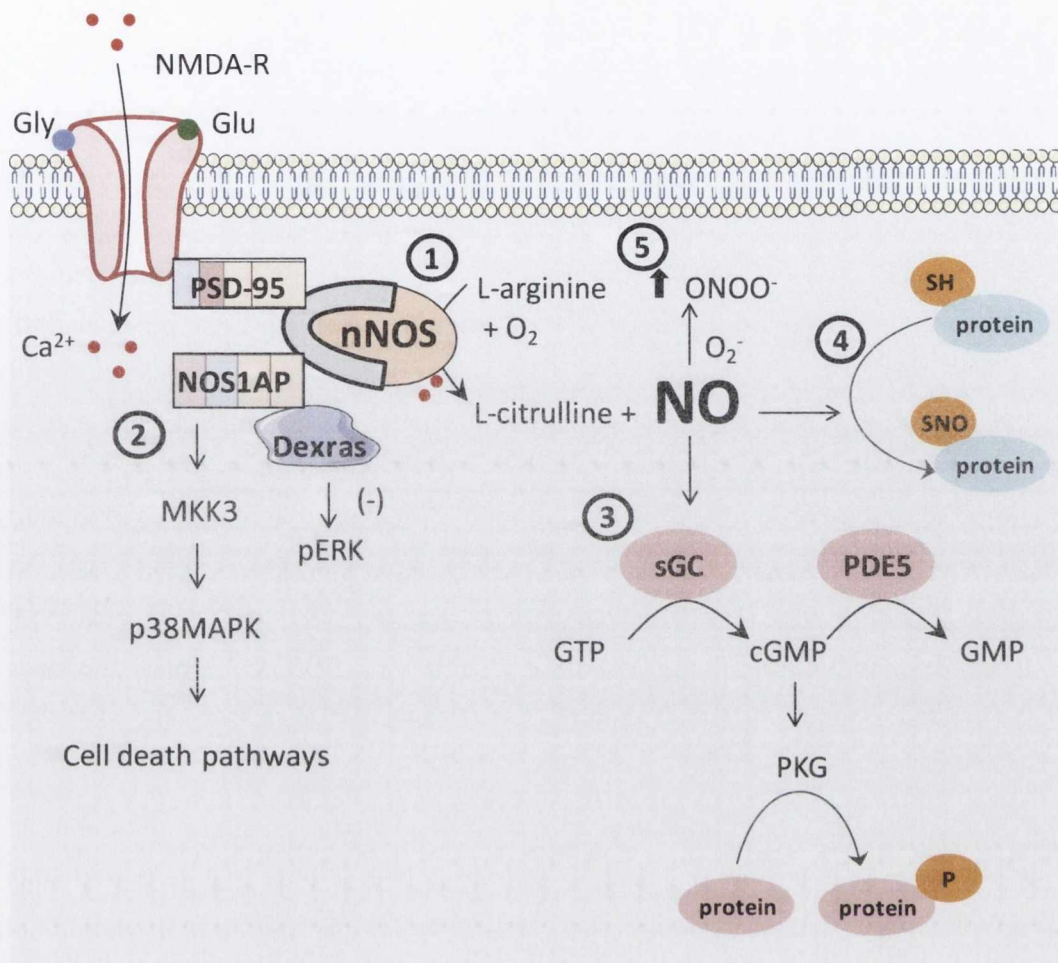


Figure 1.6: Overview of NMDA-R and NO signalling. ① The NMDA-R is coupled to nNOS via PDZ domain protein interactions with the PSD-95 scaffolding protein, thereby allowing NMDA-R-mediated Ca^{2+} influx to activate nNOS, triggering the production of NO from L-arginine and O_2 . ② nNOS also couples to NOS1AP via a PDZ domain, an interaction which has been reported to be linked to the activation of p38 MAPK pathways and the initiation of cell death pathways. NOS1AP also links to the G protein, Dexras, which has been shown to have a negative influence on the phosphorylation of important cell signalling kinase, ERK. ③ The main physiological receptor for NO is sGC, an enzyme which catalyses the conversion of GTP to cGMP. cGMP is a signalling molecule with many targets including PKG which subsequently mediates the phosphorylation of various proteins. ④ NO can also act independently of sGC and cGMP production, and in large quantities covalently bonds with protein thiol groups (protein-SH) to form S-nitroso-proteins (protein-SNO). ⑤ Large amounts of NO can also combine with the superoxide anion (O_2^-) to form the neurotoxic reactive oxygen species peroxynitrite when combined with O_2 . (For full protein names refer to List of Abbreviations, page xvi).

1.8 nNOS regulation by PDZ-binding proteins

nNOS activity can be regulated through physical interactions with several adaptor proteins, the binding of which occurs mainly via the PDZ (PSD-95/discs large/ ZO-1 homologous) domain located at the N-terminal of nNOS (**Figure 1.5**). PDZ domains refer to regions of ~90 residues which act as modules for protein-protein interactions (Scannevin and Huganir, 2000). These regions have structurally well defined pockets that can accommodate PDZ motifs that are generally found at the extreme carboxyl terminal of interacting proteins (Doucet et al., 2012). PDZ domains can also self- or hetero-dimerise, giving rise to large protein complexes. The PDZ domain of nNOS comprises of the core, or canonical, PDZ domain located at residues 1–99, and also consists of a flanking region located at residues 100–130, referred to as the internal PDZ domain (or β -finger). The PDZ domain of nNOS is known to interact with various proteins including α_1 -syntrophin, vac14, phosphofructokinase isoform M P13-kinase (PFK-M), nNOS interacting DHHC domain-containing protein (NIDD), the serotonin (5-HT) transporter (SERT) and 5HT_{2B} receptor [For review see Doucet et al. (2012)]. The nNOS PDZ domain also binds to the post-synaptic density-95 (PSD-95) and NOS1AP, which is of particular importance for the current research.

1.8.1 Post-synaptic density-95 (PSD-95)

The post-synaptic density (PSD) refers to an electron-rich membrane thickening that is found at the top of dendritic spines which functions to cluster receptors and signalling components at the synapse and allow for efficient signalling transduction pathways. The PSD forms a disk like structure approximately 200 – 800 nm wide and 30 – 50 nm thick at the post-synaptic membrane (Feng and Zhang, 2009). The region is rich in proteins including membrane receptors, cell adhesion molecules, cytoskeletal proteins and signalling proteins. The PSD family of proteins are part of a larger class of scaffolding proteins known as the membrane-associated guanylate kinase (MAGUKs), and include four proteins: PSD-93, PSD-95 and the synaptic associated proteins; SAP-97 and SAP-102. All four PSD proteins contain three PDZ domains (PDZ1-3), a SH3 (Src homology 3) domain, and a GK (guanylate kinase) domain which has lost its catalytic activity. PSD-95 plays an important role in receptor localisation and cellular signalling. It binds to over 40 proteins that can be classed as receptors, ion channels/pumps, transmembrane proteins and cytosolic proteins [For review see Doucet et al. (2012)]. Of particular interest, the first PDZ domain of PSD-95 interacts with the PDZ motif present on the extreme carboxy terminal (ct) of the NR2A/B subunit of NMDA-R. This leaves the second PDZ domain of PSD-95 (PDZ2) free to interact with nNOS, which uses its internal PDZ domain and β -finger sequence to form a ternary complex composed of NMDA-R/PSD-95 (PDZ1) and PSD-95 (PDZ2)/nNOS interactions (Christopherson et al., 1999). Taken together, this ternary complex created by PDZ domain interactions between NMDA-R, PSD-95 and nNOS acts to tether nNOS to the NMDA-R,

effectively coupling NMDA-R-induced Ca^{2+} influx to the activation of nNOS and the resulting production of NO (Guix et al., 2005) (Figure 1.7).

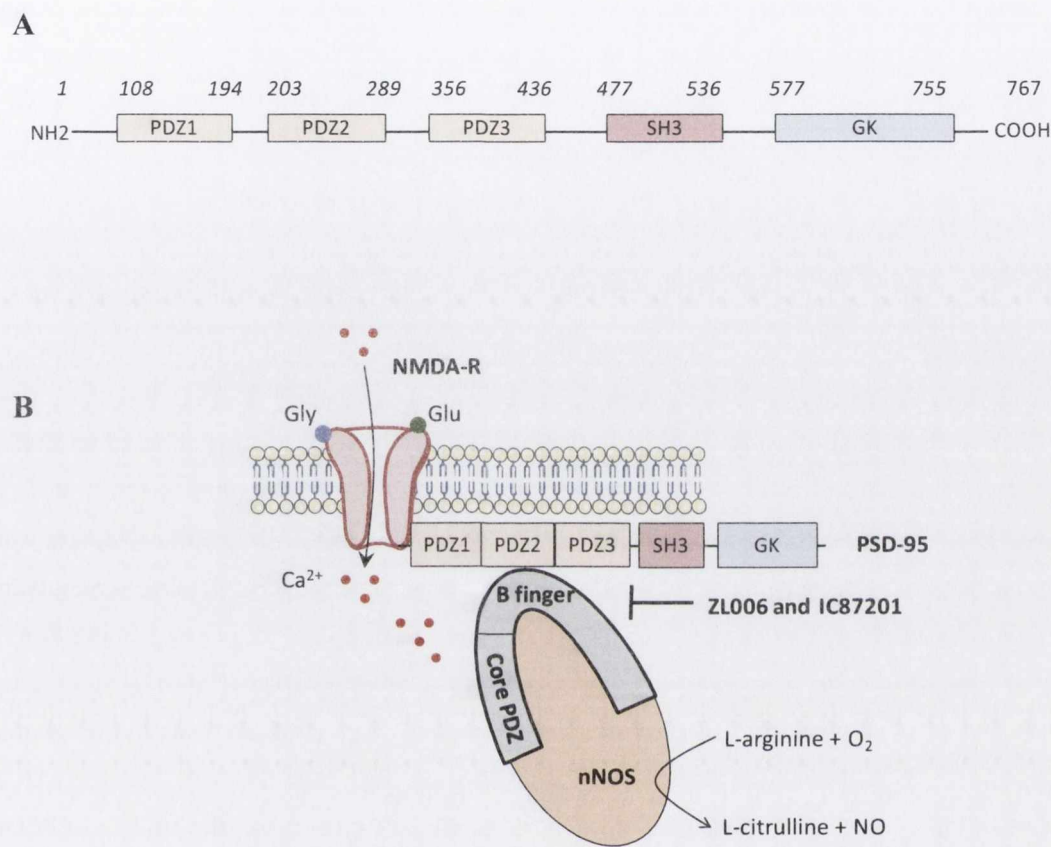


Figure 1.7: Structure of post-synaptic density-95 (PSD-95) and the NMDA-R/PSD-95/nNOS protein interaction. (A) PSD-95 contains three PDZ domains, a Src homology 3 (SH3) domain and a guanylate kinase (GK) domain, which has lost its catalytic activity. PSD-95 binds to more than 40 proteins (not shown). (B) Of particular interest the PDZ1 domain binds to the carboxyl terminal of NR2A/B subunit of NMDA-R, and the PDZ2 domain binds to the internal PDZ domain (β finger) of nNOS. These interactions form a ternary protein complex consisting of NMDA-R/PSD-95/nNOS, which tethers nNOS to NMDA-R-induced Ca^{2+} influx. Small molecule inhibitors, ZL006 and IC87201, are believed to block the PSD-95/nNOS interaction by binding to the β finger, and therefore preventing PSD-95 from interacting with nNOS (Zhou et al., 2010, Doucet et al., 2012).

1.8.2 nNOS adaptor protein 1 (NOS1AP)

nNOS adaptor protein 1 (NOS1AP) also known as CAPON [carboxy-terminal PSD95-Dlg-ZO1 (PDZ) ligand of nNOS] binds to the canonical PDZ domain of nNOS and plays a role in NO related signalling. At least two isoforms of NOS1AP have now been identified in humans – the first is NOS1AP-long (NOS1AP-L) which is a 506 aa protein produced from the expression of all 10 exons of the *nos1ap* gene. The second is NOS1AP-short (NOS1AP-S) which is a 210 aa

protein generated from the expression of only the last 2 exons of the gene. NOS1AP-L contains two functional domains – an N terminal phosphotyrosine-binding (PTB) domain as well as the C-terminal PDZ binding domain (Xu et al., 2005, Brzustowicz, 2008, Carrel et al., 2009). NOS1AP best known effector protein is Dexras1 (Fang et al., 2000) - a member of the Ras family of small G proteins induced by dexamethasone. NOS1AP acts by scaffolding nNOS to Dexras1, enabling nNOS-generated NO to activate Dexras1 by S-nitrosylation. Dexras1 has been shown to negatively regulate the phosphorylation of extracellular signal-regulated kinase (ERK), a kinase involved in various signalling pathways mediating responses such as cell growth and synaptogenesis (Zhu et al., 2014). NOS1AP has also been implicated in mediating excitotoxicity, with data demonstrating that NOS1AP interacts with p38MAPK-activating kinase (MKK3), leading to downstream cell death cascades (Li et al., 2013).

NOS1AP was originally regarded as an inhibitor of PSD-95/nNOS interaction based on findings that over-expression of NOS1AP resulted in a loss of PSD-95/nNOS complexes in transfected cells (Jaffrey et al., 1998). However, this view has been challenged with functional studies which suggest that NOS1AP may act as a mediator rather than an inhibitor of NMDA-R/NO signalling. In light of this, several conceptual models have recently been proposed that may better describe the nature of the interaction between PSD-95 and NOS1AP with nNOS (Courtney et al., 2014). These conceptual models focus on research which suggests that NOS1AP binds to the canonical PDZ domain of nNOS, while PSD-95 binds to the internal PDZ motif. The fact that NOS1AP and PSD-95 bind to discrete domains of the nNOS PDZ domain suggests that the binding of PSD-95 does not necessarily exclude or compete with NOS1AP from also binding in addition to PSD-95. Instead, it has been suggested that both PSD-95 and nNOS can bind, thereby forming a large protein complex comprising of both PDZ proteins, or alternatively, that different conformational states of the nNOS PDZ domain exists, conferring differential binding properties to the domain.

In further detail to the mechanism of NOS1AP/nNOS binding, new research now indicates that the primary interaction between NOS1AP and nNOS is created not through the PDZ motif of NOS1AP, but rather through a region upstream of the C-terminus (ct) at residues 429-431 (Li et al., 2015). This research shows that the affinity of the PDZ motif of NOS1AP for binding to nNOS is extremely low (1 mM). The authors identify a dependency on the last 3 C-terminal residues of NOS1AP (Ile-Ala-Val) in order for stable binding with nNOS to occur. This was shown by the observation that a construct lacking these residues failed to be recovered in affinity purification pull-down assays. However, in addition to this area of the ct motif (residues 504-506), the results suggest that a second region of NOS1AP between residues 400 and 431 (and particularly residues 429-431) are required for the interaction with nNOS. These residues contribute to a second domain, referred to as the ExF motif, which forms a second site of contact

between the two proteins and is regarded as crucial for the NOS1AP/nNOS interaction. The PDZ motif, having low affinity for nNOS, may therefore act to stabilise the interaction between the two proteins.

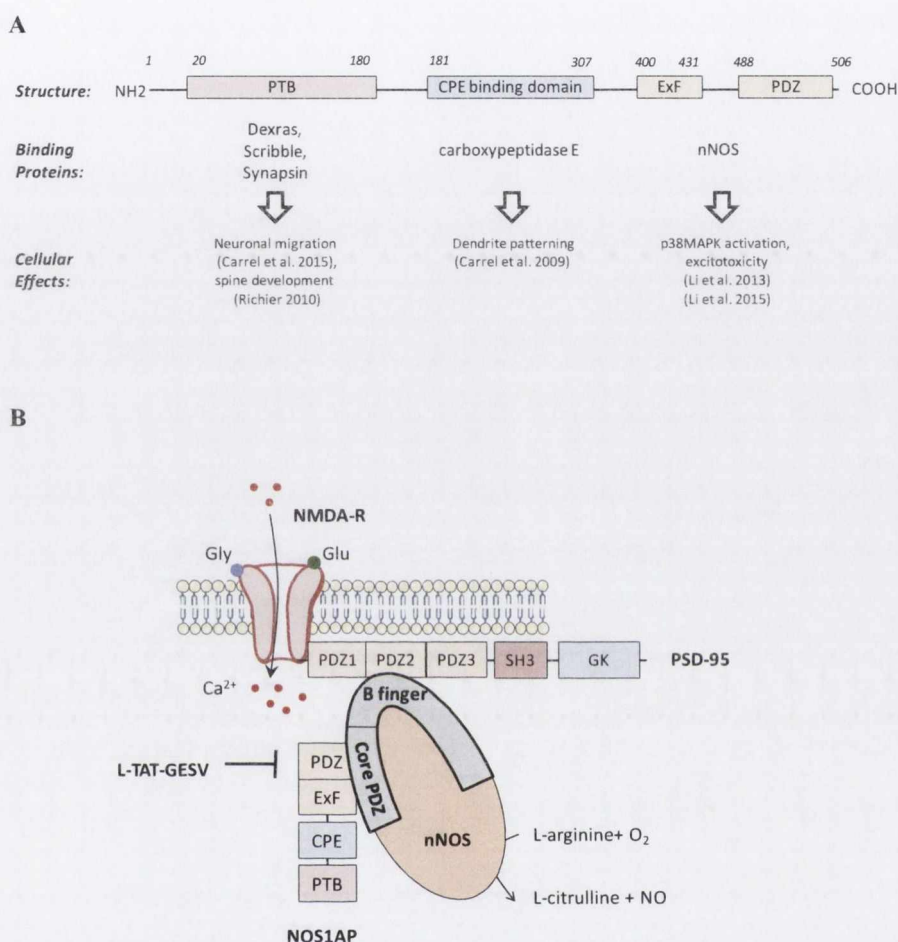


Figure 1.8: Structure of NOS1AP-L and the NOS1AP/nNOS interaction. (A) NOS1AP exists in three isomers. The longest isomer (NOS1AP-L) is a 506 aa protein and is displayed above. It contains an N-terminal phosphotyrosine binding (PTB) domain (residues 20-180) which interacts with proteins including Dexas, Scribble and Synapsin and plays a role in cortical neuronal migration and spine and synapse development. A region between 181-301 has been shown to bind to carboxypeptidase E (CPE) and is important in dendritogenesis. Two regions of NOS1AP are important for the interaction of NOS1AP with nNOS. Firstly, a novel, ExF motif between residues 400-431 has recently been identified as a crucial motif in order for binding. Secondly, the PDZ motif (residues 488-506) is also known to play an important role in stabilizing the NOS1AP/nNOS interaction. (B) The PDZ motif and ExF domain of NOS1AP interact with the canonical (core) PDZ domain of nNOS. The peptide inhibitor, L-TAT-GESV blocks this interaction by binding to the canonical (core) PDZ domain of nNOS.

1.9 Glutamate in neuronal atrophy and CNS disorders

Although glutamate neurotransmission is crucial for normal physiological functions of the CNS, excessive or sustained glutamate receptor activation is detrimental to neurons and can lead to neuronal atrophy, degeneration and excitotoxicity. Excess glutamatergic stimulation is a feature of a wide range of CNS disorders, most notably ischaemia, epilepsy, neuropathic pain, traumatic brain injury (TBI) and major depression, and is also believed to contribute to neuronal loss in neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's disease (Muir, 2006). Overactivation or prolonged stimulation of the NMDA-R is particularly involved in the detrimental effects of excessive glutamatergic stimulation, and can lead to the process of excitotoxicity through excessive Ca^{2+} influx and activation of Ca^{2+} dependent signalling molecules (Willard and Koochekpour, 2013). Excitotoxicity results in the production of reactive oxygen species (ROS) and abnormal activation of proteases and caspases - ultimately contributing to neuronal death. Glutamate-induced neuronal pathology has been reported to begin with neuronal atrophy, dendrite regression and spine loss (Monnerie et al., 2003), and several studies have investigated the effects of glutamate modulation in neurite outgrowth and dendrite morphology in this regard (**Table 1.2**). Overall, these studies indicate that glutamate may have biphasic effects on outgrowth, acting as a permissive cue for outgrowth at low concentrations, while inducing atrophy at higher concentrations. Targeting these early atrophic effects of excessive glutamate stimulation may provide a strategy to prevent subsequent cell death and promote neuronal remodelling and functional recovery in many disease states.

Table 1.2: Effects of glutamate, NO and downstream proteins on neurite outgrowth

Treatment	Culture type	Effects on neurite outgrowth	References
Glutamate (Glu)			
Glu (100 µM - 1 mM)	Hippocampal neurons	Reduced outgrowth	(Mattson et al., 1988)
Glu(1-2 mM)	Cortical neurons	Reduced outgrowth	(Esquenazi et al., 2002)
Glu (100 nM – 100 µM)	Motoneurons	Reduced dendrite outgrowth	(Metzger et al., 1998)
Glu (5 µM)	Hippocampal neurons	Reduced outgrowth	(Podestà et al., 2014)
Glu receptor agonists			
NMDA (100 µM)	Hippocampal neurons	Increased branching	(Kuo et al., 2010)
NMDA (1mM)	Cortical neurons	Reduced outgrowth	(Esquenazi et al., 2002, Monnerie et al., 2003)
KA (1 µM, 10 µM)	Hippocampal neurons	Reduced outgrowth	(Mattson et al., 1988)
AMPA agonist	Cortical neurons	No effect	(Esquenazi et al., 2002)
Glu + glu receptor antagonists			
kynurenic acid	Cortical neurons	Attenuated glu-induced reductions	(Esquenazi et al., 2002)
MK-801 (NMDA-R antagonist)	Cortical neurons	Attenuated glu-induced reductions	(Esquenazi et al., 2002)
	Hippocampal neurons	Attenuated glu-induced reductions	(Podestà et al., 2014)
DGG (ionotropic-R antagonist)	Hippocampal neurons	Attenuated glu-induced reductions	(Mattson et al., 1988)
AP5 (NMDA-R antagonist)	Hippocampal neurons	Attenuated NMDA-induced increases	(Kuo et al., 2010)
Other glu modulators			
Calcium channel blocker (Co ²⁺)	Hippocampal neurons	Attenuated glu-induced reductions	(Mattson et al., 1988)
Calmidazolium/calpain inhibitor III	Hippocampal neurons	Attenuated glu-induced reductions	(Mattson et al., 1988)
Riluzole	DRG neurons	Increased outgrowth	(Shortland et al., 2006)
Nitric Oxide (NO)			
NO donors			
3-morpholino-sydononimine (1-2mM)	DRG neurons	Inhibits growth	(Hess et al., 1993)
S-nitrosocysteine (0.4-1mM)	DRG neurons	Inhibits growth	(Hess et al., 1993)
sodium nitrite (100uM)/ SNP (100nM)	Hippocampal neurons	Increased NGF-induced outgrowth	(Hindley et al., 1997)
Other NO modulators			
unconjugated bilirubin (increases NO)		Reduced outgrowth	(Silva et al., 2012)
haemoglobin (binds NO)	Hippocampal neurons	Attenuated NO + NGF effects	(Hindley et al., 1997)
8-Br-cGMP (cGMP analog)	Hippocampal neurons	Mimicked NO+ NGF effects	(Hindley et al., 1997)
methylene blue/LY83583 (sGC inhibitor)	Hippocampal neurons	attenuated NO + NGF effects	(Hindley et al., 1997)
PSD-95			
PSD-95 overexpression	Hippocampal neurons	Decreased branching	(Charych et al., 2006)
		Increased dendrite number & dendritogenesis	(Charych et al., 2006, Bustos et al., 2014)
PSD-95 knockdown	Hippocampal neurons		(El-Husseini et al., 2000)
PSD-95 overexpression	Hippocampal neurons	Increases dendrite spine number & size	
NOS1AP			
NOS1AP-L overexpression at DIV 0- 12	Cortical neurons	Reduced dendrite number	(Carrel et al., 2009)
NOS1AP-L knockdown at DIV 0- 12	Cortical neurons	Increased dendrite number	(Carrel et al., 2009)
NOS1AP-S overexpression at DIV 5-7	Cortical neurons	Reduced dendrite number	(Carrel et al., 2009)
NOS1AP (PTB domain-dependent)	Hippocampal neurons	Increased dendritic protrusions	(Richier et al., 2010)

Abbreviations: 8-Br-cGMP: 8-Bromoguanosine 3',5'-cyclic monophosphate, AP5: 2-amino-5-phosphonopentanoate, cGMP: cyclic guanosine monophosphate, DGG: D-Glutamylglycine, DIV: Days *in vitro*, DRG: Dorsal root ganglion, Glu: Glutamate, KA: kainic acid, NGF: Nerve growth factor, NO: nitric oxide, PTB: Phosphotyrosine binding domain, SNP: Sodium Nitroprusside.

1.10 NMDA-R antagonists for CNS injury and disease

Due to the detrimental effects caused by overactivation of iGluRs - and NMDA-Rs in particular - there has been considerable interest in the therapeutic potential of NMDA-R antagonism for the prevention and treatment of brain injury and disease. NMDA-R antagonism can be achieved by receptor antagonists which block the ion channel phencyclidine (PCP) site such as ketamine, MK-801, PCP and memantine, as well as blocking both the glutamate binding site with antagonists including selfotel, and the glycine site by agents including kynurenic acid. Targeting NMDA-R as a therapeutic strategy has been explored in fields of research including stroke, epilepsy, TBI, neuropathic pain, schizophrenia and major depression (Ikonomidou and Turski, 2002, Konradi and Heckers, 2003, Muir, 2006, Maeng and Zarate, 2007, Zhou et al., 2010). In terms of major

depression, NMDA-R antagonists are known to have antidepressant properties, and most notably, a sub-anaesthetic dose of ketamine (0.5mg/kg) produces a rapid and robust antidepressant response in patients within hours of administration (Berman et al., 2000, Zarate et al., 2006, DiazGranados et al., 2010). Interestingly, compelling evidence now suggests that ketamine may elicit its antidepressant effects through increasing synaptogenesis. For example, ketamine administration has been shown to rapidly activate the mammalian target of rapamycin (mTOR) signalling pathway in the PFC of rats, accompanied by increased production of synaptic signalling proteins, leading to an increased number and function of new spine synapses in the PFC (Li et al., 2010). Such results suggest that NMDA-R antagonists possess neurotrophic effects which may contribute to their therapeutic effects, and opens up novel possibilities for the use of NMDA-R antagonists for the prevention of neuronal atrophy and the promotion of neuronal remodelling.

However, despite the efficacy of several NMDA-R antagonists in providing therapeutic relief in disease states, their use in the clinic is limited due to their adverse side effects which include psychomimetic disturbances and nausea (Muir, 2006, Doucet et al., 2012). In addition, NMDA-R activation is required for the normal function of the CNS, and total blockade of NMDA-R function is therefore not desired. Indeed, the repeated failure of NMDA-R antagonists in clinical trials for ischaemic stroke and TBI are thought to be due in part to the fact that NMDA-R activation is required for essential neuronal excitation (Aarts et al., 2002). The total antagonism of NMDA-R prevents any pro-survival effects that NMDA-R activation may have on cell recovery, such as NMDA-R-mediated activation of cAMP response-element binding protein (CREB) – a transcription factor that is known to elicit pro-survival effects and stimulate the production of neurotrophins (Ikonomidou and Turski, 2002). Although excitotoxic events are believed to occur as a result of NMDA-R-mediated Ca^{2+} influx, it has been shown that Ca^{2+} blockers alone are not sufficient to block excitotoxic insults, with findings indicating that these blockers fail to provide neuroprotection following ischaemic stroke (Horn and Limburg, 2001). For these reasons, research is aimed at targeting downstream of the NMDA-R. Such a strategy is hoped to harness the protective and/or neurotrophic potential of NMDA-R antagonists without incurring the detrimental effects associated with their use, and represents a more promising option for the development of novel drug targets.

1.11 nNOS inhibitors for CNS injury and disease

The generation of excess NO via NMDA-R activation and Ca^{2+} influx is widely implicated in contributing to glutamate excitotoxicity (Dawson et al., 1991), and increased NO concentrations have been reported in several neurodegenerative diseases including Alzheimer's and Parkinson's disease, ischaemic stroke and depression (Iadecola, 1997, Wegener and Volke, 2010, Dhir and

Kulkarni, 2011, Labby et al., 2012). For example, NO levels increase dramatically (to micromolar levels) within minutes following an ischaemic insult. This increase in NO concentration contributes to oxidative damage, energy failure and DNA damage. As a result, nNOS inhibitors have been assessed for their ability to provide protection against excitotoxic insults and neurodegeneration. Initial observations have shown that inhibition of NO synthesis can attenuate NMDA-induced neurotoxicity in neuronal cultures and reduce brain damage produced by middle cerebral artery occlusion (MCAO) in mice (Iadecola, 1997), indicating that NO is involved in the mechanisms of cerebral ischaemia. In addition, nNOS inhibitors such as NG-nitro-L-arginine (L-NA) and 7-nitroindazole (7-NI) have been shown to possess antidepressant-like effects in preclinical paradigms such as the rat and mouse forced swimming test (FST) (Harkin et al., 1999, Harkin et al., 2004). The FST is the most widely employed screening test for antidepressants in rodents and is based on the development of immobility in response to an inescapable stressor, which represents a state of 'behavioural despair'. L-NA and 7-NI dose dependently reduce immobility time and increase escape-orientated swimming behaviour, and this effect occurs in the absence of producing locomotor stimulatory effects, suggesting that they may show more clinical potential than NMDA-R antagonists (Harkin et al., 1999, Wegener and Volke, 2010). Despite these promising leads, nNOS inhibitors have not been approved for clinical use, and the development of highly selective nNOS inhibitors has proven difficult and has limited the development of such drugs (Doucet et al. 2012). In addition, nNOS is expressed in other tissues other than the brain - where it regulates physiological functions including skeletal muscle contraction and sphincter muscle relaxation in the gut - meaning that nNOS inhibition can lead to off-target gastrointestinal and genitourinary side effects (Vallance and Leiper, 2002).

1.12 Specific inhibition of protein interactions in the NMDA-R/NO signalling pathway

Since direct NMDA-R antagonism and nNOS inhibition are both linked to adverse side effects, more recent research aims to selectively disrupt specific elements downstream of NMDA-R activation through the use of cell penetrating peptide (CPP) inhibitors or small molecular weight inhibitors. The use of such inhibitors represents a means to selectively target specific protein-protein interactions in a cell pathway while leaving receptor and enzymatic activity unaffected, and thus aims to circumvent adverse side effects associated with direct NMDA-R antagonism or nNOS inhibition (Vaslin et al., 2011). CPPs are composed of a biologically aa sequence (termed the cargo) - which is based on the protein interaction of interest - attached to a cell transduction domain which mediates internalisation through endocytosis or direct translocation mechanisms (**Figure 1.9**) (Bechara and Sagan, 2013). The transduction domain most commonly used is the 10 aa human immunodeficiency virus (HIV) trans-activating transcriptional activator (TAT)

transporter sequence (TAT48-57) (GRKKRRQRRR) which confers cell permeability to the cargo by virtue of its short length and efficacy of cell membrane translocation (Borsello and Bonny, 2004). CPPs are of particular interest for the inhibition of elements downstream of the NMDA-R for the treatment of ischaemia and the prevention of excitotoxicity (Aarts et al., 2002, Borsello et al., 2003). High throughput screening approaches have also identified small molecular weight inhibitors which readily cross cell membranes and inhibit specific protein interactions downstream of NMDA-R activation. These small-molecule inhibitors are regarded as the optimal means of specific protein inhibition in a clinical setting because they bypass problems associated with the delivery of biologically active peptides that are subject to rapid degradation, which limits their activity *in vivo* (Doucet et al., 2012). Given the essential roles of NMDA-R in normal brain function, such targeted inhibition of protein interactions represents a promising strategy to provide potential therapeutic effects without the adverse effects associated with direct NMDA-R or nNOS inhibition (Aarts et al., 2002, Cao et al., 2005, Florio et al., 2009, Zhou et al., 2010, Doucet et al., 2012, Doucet et al., 2013).

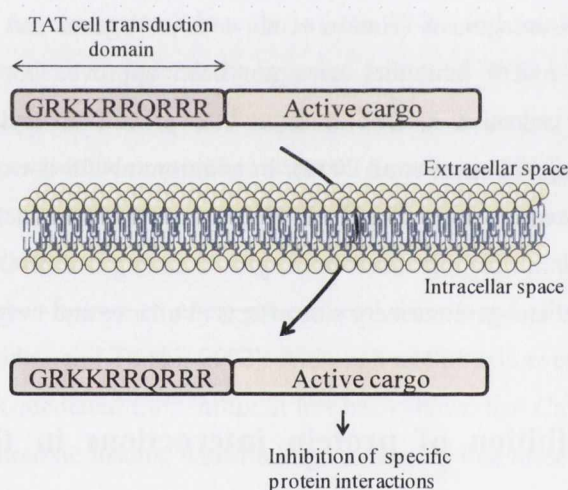


Figure 1.9: Cell penetrating peptides (CPPs). CPPs are composed of a biologically active amino acid (aa) sequence (termed the cargo) fused to a cell transduction domain - most commonly, the HIV-TAT transporter sequence (GRKKRRQRRR). The cargo is capable of binding to specific aa sequences on target proteins, and CPPs can therefore selectively uncouple protein-protein interactions for the prevention of particular cell signalling pathways.

1.12.1 Peptide inhibitors of NMDA-R/PSD-95/nNOS

Disruption of the interaction between NMDA-R and PSD-95 has been achieved through the development of a peptide comprising the last nine ct residues of NR2B fused to the cell membrane transduction domain of the HIV-type 1 (HIV-1) TAT protein to create the cell membrane-permeable TAT-NR2B9c. This peptide competitively blocks the NMDA-R from interacting with PSD-95 while maintaining NMDA-Rs ability to participate in synaptic activity

and Ca^{2+} influx (Aarts et al., 2002). TAT-NR2B9c peptide has shown neuroprotective properties against excitotoxic insults *in vitro*, and reduced focal ischaemic brain damage as well as improving neurological scores in the acute phase in a middle cerebral artery occlusion (MCAO) rat model of stroke *in vivo* (Aarts et al., 2002, Sun et al., 2008). TAT-NR2B9c has also been shown to reduce damage and provide neuroprotection in primate models of stroke (Cook et al., 2012) and most importantly, this peptide has been reported to reduce ischaemic infarcts in a phase 2 clinical trial with patients receiving endovascular repair for intracranial aneurysm (Hill et al., 2012). A more recent study also demonstrated the efficacy of delayed administration of the recombinant chimeric peptide TAT-HA-NR2B9c (comprising of TAT-NR2B9c fused to influenza virus hemagglutinin epitope-tag) in the subacute phase after MCAO. Interestingly, this study reported that functional recovery in this rodent model was accompanied by increases in neurogenesis in the dentate gyrus and increased spine density of mature neurons in the motor cortex (Zhou et al., 2015), indicating that disruption of NMDA-R/PSD-95 promotes functional recovery through the promotion of neuronal plasticity.

Specific inhibition of the PSD-95/nNOS interface also been achieved using cell permeable fusion proteins based on the first 300 residues of nNOS, as an alternative method of reducing NMDA-R downstream signalling. Firstly, studies by Cao et al. (2005) developed an nNOS₁₋₃₀₀ protein fragment which contains the canonical PDZ domain and β -finger of nNOS and can therefore act to competitively inhibit nNOS from interacting with the PDZ2 domain of PSD-95. This protein fragment prevented the induction of stress-activated protein kinase p38 and glutamate-induced neuronal death. In addition, a similar TAT protein fragment comprising of the first 299 residues (nNOS₁₋₂₉₉) has been reported to disrupt the PSD-95/nNOS interaction *in vitro* with an IC_{50} of 300 nM and showed efficacy in inhibiting acute thermal hyperalgesia and chronic mechanical allodynia in a rodent model of neuropathic pain (Florio et al. 2009). Furthermore, lentiviral-mediated overexpression of nNOS₁₋₃₀₀ in the right cerebral cortex of MCAO mice was capable of significantly reducing the infarct area and improved neurological scores (Zhou et al. 2010).

1.12.2 Small-molecule inhibitors of PSD95/nNOS interaction: ZL006 and IC87201

In addition to peptide inhibitors, small molecular weight compounds have also been developed which inhibit the interaction of PSD-95 with nNOS. Firstly, IC87201 (2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl)-4,6-dichlorophenol) was identified in a high throughput screen and was found to disrupt the PSD-95/nNOS interaction with an IC_{50} of 31 μM without inhibiting nNOS catalytic activity (Florio et al. 2009). Intrathecal administration of IC87201 in a mouse model of NMDA-induced thermal hyperalgesia resulted in antinociceptive effects at a dose of 1 pmol as analysed by a reduction in warm water tail flick latency. In addition, IC87201 was also effective after intraperitoneal injection with an EC_{50} of 0.1 mg/kg. IC87201 has also shown

efficacy in abolishing mechanical allodynia after intrathecal (at 50 and 100 nmol dose) and intraperitoneal (2mg/kg) administration in a rat model of sciatic nerve constriction, indicating that IC87201 is capable of crossing the blood brain barrier (BBB) (Florio et al., 2009).

A structurally related molecule was later identified, called ZL006 (4-(3,5-dichloro-2-hydroxybenzylamino)-2-hydroxybenzoic acid) (Zhou et al., 2010). This compound has been shown to inhibit NMDA-R-dependent NO synthesis in cortical neurons with an IC_{50} of 82 nM and is also able to cross the BBB after systemic administration. ZL006 (1.5 mg/kg) has shown preclinical efficacy in a MCAO model of stroke in mice, without affecting nNOS expression or catalytic activity, or NMDA-R function in these studies. In addition, the canonical PDZ domain interactions of nNOS, for example the NOS1AP/nNOS interaction, are not inhibited by ZL006. Moreover, behavioural measures associated with NMDA-R antagonism such as spatial memory and aggressive behaviour are not affected by ZL006, indicating that this molecule disrupts PSD-95/nNOS without causing adverse effects and lends support to its potential for drug development. More recent research further confirms the efficacy of ZL006 in MCAO models and demonstrates that dissociating PSD-95 from nNOS using ZL006 (1.5mg/kg/d, i.v.) enhances neuronal survival and promotes migration of new neurons into the injury area, and increases neurite outgrowth in newborn neurons and dendritic spine formation in mature neurons in the ischaemic brain (Luo et al., 2014). Furthermore, recent data indicate the utility of these small molecules as novel analgesics, and show that both ZL006 and IC87201 possess antinociceptive effects in animal models of inflammatory and neuropathic pain without the adverse effects of NMDA-R antagonists (Lee et al., 2015). Both IC87201 and ZL006 have also demonstrated neuroprotective effects against 1-methyl-4-phenyl-pyridinium (MPP^+)-induced neuronal injury and apoptotic cell death in an *in vitro* model of Parkinson's Disease (Hu et al., 2014).

Recent data from our laboratory indicates that both IC87201 (0.01–2 mg/kg) and ZL006 (10 mg/kg) lead to antidepressant-like effects by reducing immobility time in the FST and tail suspension test (TST) following a single intraperitoneal administration in mice (Doucet et al., 2013). These effects are novel in their mode of action in that they become apparent 24 h and 72 h following drug administration in a modified version of the FST and TST, which is dependent on pre-test exposure to the behavioural tests. These effects are in contrast to the acute behavioural effects observed with the classical antidepressant imipramine which are observed 1h following drug administration. Similar delayed and sustained effects were observed with a single administration of TRIM (50mg/kg) and ketamine (30 mg/kg) in the TST following a pre-test exposure. The novel delayed and sustained antidepressant effects observed with inhibitors of the NMDA-R/PSD-95/nNOS interaction may be indicative of adaptive changes to neural plasticity.

IC87201 did not display anxiolytic effects as assessed by subjecting mice to the light dark box and elevated plus maze.

ZL006 was developed through a rationalised drug design approach based on the recognition that an ionic interaction between two nNOS internal PDZ domain (β finger) residues, Asp62 and Arg121, are structurally required for the interaction of nNOS to the PDZ2 of PSD-95. This is supported by the fact that mutation of Arg-121 has been shown to destabilise the nNOS β -finger and lead to a loss of its structure, preventing nNOS-PDZ from binding to PSD-95-PDZ2, thereby showing that Arg-121 is necessary for the PSD-95/nNOS interaction. The mechanism of action of ZL006 is therefore believed to involve direct binding to the β -finger of nNOS by forming an ionic bond between the carboxyl group of ZL006 and the amino group in Arg-121 nNOS β finger, and also a hydrophobic bond between the hydrophobic ring of ZL006 and Leu-107 or Phe-111 of nNOS, thereby interrupting the conformational change of nNOS PDZ domain (Zhou et al. 2010). IC87201 has a similar structure to ZL006 and has been suggested to act in the same way, although the exact binding site for IC87201 has not yet been established (**Figure 1.10**). Recent studies using fluorescence polarisation (FP), isothermal titration calorimetry (ITC), and nuclear magnetic resonance techniques suggest that the mode of action of ZL006 and IC87201 may not involve direct binding to the β finger (Bach et al., 2015), highlighting the need for further characterisation of the mechanism of action of these molecules.

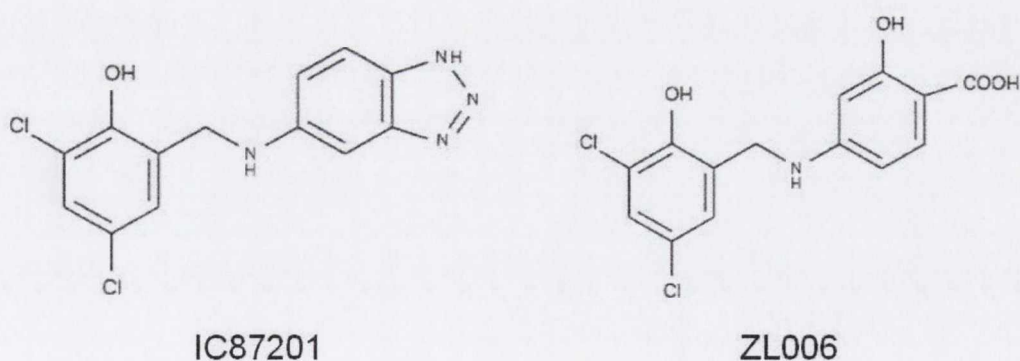


Figure 1.10: Structure of IC87201 and ZL006. 2-((1H-benzo[d] [1,2,3]triazol-5-ylamino) methyl)-4,6-dichlorophenol (IC87201) and 4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid (ZL006) are structurally related small-molecule inhibitors of the PSD-95/nNOS interface.

1.12.3 Peptide and small-molecule inhibitors of the NOS1AP/nNOS interaction

In addition to the PSD-95/nNOS interaction, the interface between NOS1AP and nNOS has also been targeted for its potential to provide protection against excessive glutamatergic stimulation (Li et al., 2013). The NOS1AP/nNOS interface has been successfully inhibited *in vitro* and *in vivo* by the use of a specifically designed TAT peptide, designated L-TAT-GESV (Li et al., 2013, Li et al., 2015). This peptide binds to the canonical PDZ domain of nNOS which binds to NOS1AP, thereby competing with NOS1AP for binding. L-TAT-GESV has been shown to prevent NMDA-induced recruitment of NOS1AP and produces neuroprotective effects *in vivo* in a model of neonatal hypoxia-ischaemia in rats. As mentioned previously, a novel region of NOS1AP comprising residues 400-431 and termed the ExF motif was recently identified as crucial for the NOS1AP/nNOS interaction (Li et al., 2015). In line with this, a peptide construct consisting of only the ExF motif (residues 400-503), and lacking the PDZ motif of NOS1AP, was found to be as effective in protecting against NMDA-induced p38 MAPK activation as a peptide construct containing only the PDZ motif, showing that this ExF motif may represent a second pharmacological site on NOS1AP for potential drug development (Li et al., 2015).

Interestingly, disruption of the interaction between NOS1AP and nNOS has also been shown to provide anxiolytic effects in animal models (Zhu et al., 2014), indicating that this pathway may also be involved in stress related illnesses. In this study, overexpression of NOS1AP in the hippocampus of mice promoted anxiety-like behaviours, while disruption of NOS1AP/nNOS by over-expressing peptides designed from ct amino acids of NOS1AP was capable of producing anxiolytic effects. These effects were also observed in response to delivery of TAT-CAPON-12C (a peptide comprising TAT and the 12 ct amino acids of NOS1AP) to the hippocampus of mice, as well as through administration of a small-molecule inhibitor of the NOS1AP/nNOS interface, ZLc-002. The authors show that mice exposed to chronic mild stress (CMS) exhibit increased NOS1AP/nNOS coupling in the hippocampus concurrent with displaying anxiety-like behaviours, and that disruption of NOS1AP/nNOS can ameliorate these CMS-induced anxiety-like behaviours. Dexras1-ERK signalling was found to be involved in mediating the anxiolytic effects of these nNOS-NOS1AP blockers.

1.13 Mitogen-Activated Protein Kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) are serine/threonine specific kinases involved in a wide range of physiological functions including cell proliferation, synaptic plasticity and apoptosis (Pearson et al., 2001, Thomas and Huganir, 2004, Cargnello and Roux, 2011). MAPKs operate as part of a phosphorelay system that consists of three tiers of sequentially activated kinases which phosphorylate and activate one another (Johnson and Lapadat, 2002, Roux and

Blenis, 2004) (**Figure 1.11**). These three tiers are known as MAPKs, MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs). MAPKKKs phosphorylate and activate specific MAPKKs and these activated MAPKKs go on to phosphorylate and activate specific, multifunctional MAPKs. MAPKs can be divided into four modules comprising of the extracellular signal-related kinases (ERK) modules (ERK1/2 and ERK5), and the stress-activated protein kinases (SAPKs) which include the c-Jun NH₂-terminal kinases (JNK1, JNK2 and JNK3) and the p38 enzymes (p38 α , p38 β , p38 γ and p38 δ). The ERK pathways are activated by stimuli such as mitogens and growth factors including epidermal growth factor (EGF) and fibroblast growth factor (FGF), and activation of these pathways are associated with responses such as cell proliferation and differentiation (Nishimoto and Nishida, 2006). In contrast, JNK and p38 MAPK are activated in response to stressors such as excitotoxicity and oxidative stress as well as inflammatory cytokines and lead to inflammatory and apoptotic cellular responses (Cargnello and Roux, 2011).

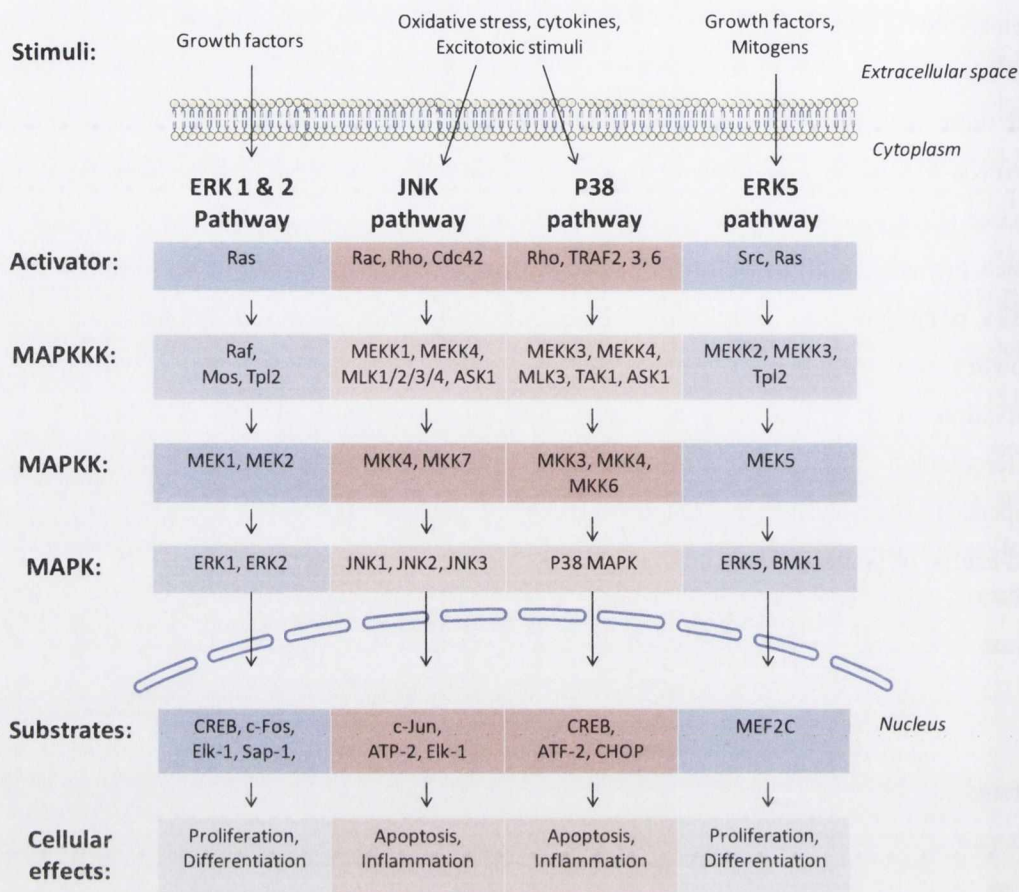


Figure 1.11: Mitogen-Activated Protein Kinase (MAPK) signalling pathways. MAPKs are serine/threonine specific kinases which operate as part of a phosphorelay system consisting of three tiers of sequentially activated kinases - MAPK kinase kinases (MKKKs), MAPK kinases (MKKs) and MAPKs. There are four characterised branches of MAPKs comprising the extracellular signal-related kinases (ERK1 & 2 and ERK5) (*blue shading*), as well as the stress-activated protein kinases (SAPKs) comprising of the c-Jun NH2-terminal kinases (JNK) and the p38 enzyme pathways (*red shading*). ASK1 = Apoptosis signal-regulating kinase 1, ATF-2 = Activating transcription factor 2, BMK1 = Big MAPK 1, Cdc42 = Cell division control protein 42, CREB = Cyclic-AMP responsive-element-binding protein 1, MEF2C = Myocyte enhancer binding factor 2C, Sap-1 = SRF accessory protein-1, TAK1 = TGF β -activated kinase 1, Tpl2 = Tumour progression locus 2, TRAF = TNF receptor associated factor.

1.13.1 JNK inhibition to protect against NMDA-R-mediated neuronal damage

Activation of JNK is widely associated with excess NMDA-R activation and subsequent excitotoxic cell responses. Inhibition of the JNK signalling pathway in neurons has therefore been investigated for its potential to provide neuroprotection in diseases associated with overactivation of NMDA-Rs and excitotoxicity such as ischaemia, epilepsy and neuropathic pain. JNK activation represents the final stage of a multi-step signalling cascade before the phosphorylation of transcription factors, and so targeting it may lengthen the duration of the therapeutic window,

which would be of particular significance in pathological illnesses such as ischaemia (Borsello et al., 2003, Borsello and Bonny, 2004). Disruption of JNK by both genetic and pharmacological approaches has been shown to offer neuroprotection against excitotoxicity. For instance, neurons from mice in which the *Jnk3* gene is disrupted are reportedly more resistant to excitotoxic cell death (Yang et al., 1997). Of particular interest is a cell-penetrating, protease-resistant peptide inhibitor of JNK referred to as D-JNKI-1. This peptide acts to selectively prevent JNK from interacting with its main substrate, c-Jun by competitively preventing the access of JNK to c-Jun. The peptide was developed by linking the 10 aa HIV TAT(48-57) transporter sequence to the 20 aa JNK binding motif of JNK-interacting protein-1/islet-brain 1 (JIP-1/IB1), a protein which shares a similar binding motif to c-Jun except that JNKs affinity for binding to JIP-1/IB1 is reported to be 100 fold higher than that of its affinity for c-Jun (Bonny et al., 2001, Borsello et al., 2003).

D-JNKI-1 has been shown to provide neuroprotection in animal models of stroke (Borsello et al., 2003), kainic acid-induced seizures (Zhao et al., 2012), mechanical allodynia and neuropathic pain (Zhuang et al., 2006, Gao et al., 2009) as well as in hearing loss (Lynch and Kil, 2005, Eshraghi et al., 2006). Firstly, Borsello et al. (2003) evaluated the neuroprotective properties of D-JNKI-1 in MCAO models of stroke in mice. D-JNKI-1 (2 μ M) completely prevented increases in c-Jun after NMDA-induced (100 μ M) activation of the JNK pathway and protected cultured neurons against NMDA-induced excitotoxicity (Borsello et al., 2003). *In vivo*, the authors report that intraventricular administration of D-JNKI-1 6 h after transient occlusion in adult mice was capable of reducing lesion volume by more than 90% for at least 14 days while also preventing behavioural consequences of MCAO. In a second model of MCAO, which involved permanent occlusion in 14 day old rat pups, D-JNKI-1 was systemically administered and was found to reduce lesion volume by 78% and 48% at 6 h and 12 h after ischaemia respectively. These protective effects correlated with a prevention in the increase of c-Jun phosphorylation and c-Fos transcription. These results indicate that inhibition of JNK by D-JNKI-1 provides neuroprotection with a long therapeutic window and therapeutic benefits in terms of lesion size as well as functional outcomes in rodent models of stroke. In addition, research by Zhao et al. (2012) showed that kainic acid-induced seizures increased the activation of JNK1, JNK3 and their activator, MKK4 in brain mitochondria, and D-JNKI-1 prevented apoptotic events associated with JNK signalling including cytochrome c release and Poly (ADP-ribose) polymerase (PARP) cleavage.

1.13.2 p38 MAPK inhibition to protect against NMDA-R-mediated neuronal damage

p38 MAPK is activated in response to stressors such as inflammatory cytokines, hormones, heat shock and osmotic shock, and its activation plays an important role in modulating the immune response as well as mediating cell death. Of particular importance, p38 MAPK is activated in response to glutamate, and nNOS inhibitors are capable of reducing glutamate-induced p38 MAPK activation (Cao et al., 2005). Interestingly, recent research has identified a requirement for NOS1AP in the NMDA-R mediated activation of the p38 MAPK pathway (Li et al., 2013). These authors reported that NOS1AP activation recruits p38 activating kinase, MKK3, leading to activation of p38 signalling and subsequent cell death. In line with this, NMDA-R-induced activation of the p38 MAPK pathway and cell death was reduced through the use of the NOS1AP/nNOS interacting inhibitor, L-TAT-GESV, as well as by NOS1AP/nNOS knockdown using NOS1AP-targeting small interfering RNA (siRNA). p38 MAPK is most frequently inhibited by small cell permeable compounds including the pyridinyl imidazole SB203580 and the diaryl urea compound BIRB796 (a.k.a. doramapimod). SB203580 inhibits the P38 α and p38 β isoforms and acts by inserting into the ATP binding site of p38 and therefore directly competing with ATP (Cuenda and Rousseau, 2007). While numerous stimuli that activate p38 MAPK also activate JNK pathways, the residue on the ATP binding site of p38 isoforms which binds SB203580 is well conserved meaning that SB203580 is a specific inhibitor of p38, and does not affect JNK activity. BIRB796 inhibits all isoforms of p38. Unlike SB203580, it acts as an allosteric inhibitor which interacts with residues in the kinase specificity region to induce a conformational change (Kuma et al., 2005).

1.14 Astrocytes in CNS injury and disease

Astrocytes are the most abundant glial cell in the brain and are heterogeneous cells which carry out a multitude of functions crucial to normal CNS health including the regulation of brain metabolism, ionic homeostasis and maintaining the integrity of the BBB [for a detailed review see Sofroniew and Vinters (2010)]. Although astrocytes were initially regarded as merely providing structural and homeostatic support for neurons, research carried out over the last 20 years has shown that these cells are active and dynamic participants in many CNS processes such as neurotransmission and information processing in neural circuits (Perea et al., 2009, Sofroniew and Vinters, 2010). In addition, convincing evidence has emerged implicating astrocytes as key players in processes such as synaptogenesis, synaptic plasticity and neuronal remodelling during both development and adulthood (Allen and Barres, 2005, Clarke and Barres, 2013). This re-evaluation of the emerging roles of astrocytes corresponds with increasing data in the literature linking astrocyte dysfunction to many CNS diseases associated with neuronal atrophy. In this regard, several thorough reviews can be referred to which document a prominent astroglial

pathology in several diseases including Alzheimer’s and Parkinson’s disease (Maragakis and Rothstein, 2006, Halliday et al., 2011) in addition to chronic pain (Watkins and Maier, 2003, Scholz and Woolf, 2007, Gao and Ji, 2010) ischaemic stroke (Anderson et al., 2003, Panickar and Norenberg, 2005, Takano et al., 2009) and psychiatric illnesses such as depression (Cotter et al., 2001, Rajkowska and Miguel-Hidalgo, 2007).

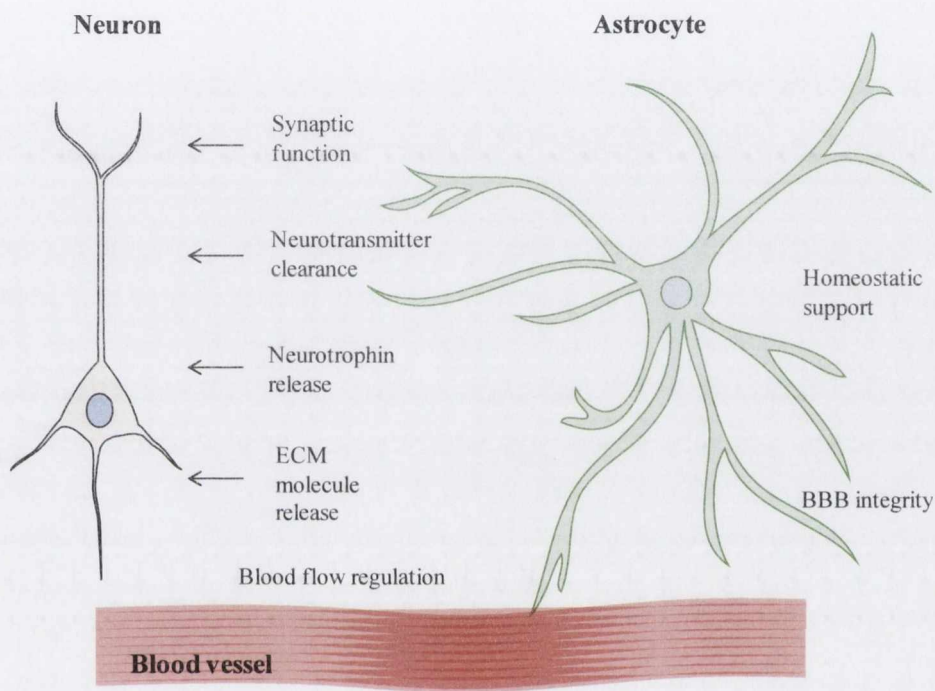


Figure 1.12: Overview of the main functions of astrocytes. Astrocytes are heterogeneous glial cells performing multiple functions crucial to the maintenance of a healthy CNS. In addition to carrying out homeostatic roles in BBB integrity and blood flow regulation, these cells are also known to be active participants in the regulation of neuronal and synaptic function. Astrocytes are crucial to neurotransmitter (particularly glutamate) clearance and are known to secrete neurotrophic factors and ECM molecules which can impact upon neuronal survival, growth and migration.

1.14.1 The role of astrocytes in neurite outgrowth

The predominant research in the literature concerning the role of astrocytes in neurite outgrowth relates to the contribution of reactive astrocytes in the prevention of axonal regeneration and functional recovery following CNS trauma. In this regard, astrocytes are known to create a dense, fibrous glial scar composed of ECM molecules which, on one hand, provides protection by limiting the entry of potentially harmful infectious agents into the damaged area, but on the other hand, secretes inhibitory factors which prevent axonal growth (Lefrançois et al., 1997, Tuinstra et al., 2013, Hashemian et al., 2014, Snow and Beller, 2014). Several *in vitro* studies have

investigated the factors secreted by reactive astrocytes which account for their negative effects on axonal growth, and a major contributor has been identified as a class of ECM molecule called sulphate proteoglycans (Snow et al., 1990, McKeon et al., 1991, Smith-Thomas et al., 1994). Other investigations have identified growth promoting factors produced by reactive astrocytes which may aid axonal regeneration following spinal cord injury (Chang et al., 2013), thereby highlighting the highly heterogeneous roles played by reactive astrocytes in the damaged CNS (Liu et al., 2015).

Aside from the role of reactive astrocytes in axonal regeneration after trauma, astrocytes in the healthy CNS are known to actively secrete trophic factors into their environment which promote neuronal growth and development (Barres, 2008, Allen, 2013). The ability of astrocytes to synthesise trophic factors that enhance neuronal growth was recognised over 25 years ago (Assouline et al., 1987), and as such, astrocytes are regularly used *in vitro* as a substrate to provide support for neuronal cultures. More recently, media derived from astrocytes – termed conditioned media (CM) - has been shown to enhance the branching of dendrites (Previtera et al., 2010). Interestingly, several reports indicate that CM derived from astrocytes is sufficient to protect neurons against a range of deleterious stimuli. For instance, recent data has shown that astrocyte CM attenuates against glutamate-induced apoptotic cell death in rat spinal cord neurons (Lu et al., 2015). In addition, CM from astrocytes has also recently demonstrated neuroprotective effects against the environmental chemical, methylmercury, an effect attributed to the release of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Takemoto et al., 2015). Furthermore, astrocyte CM is also capable of protecting primary cultures of hippocampal neurons from corticosterone-induced damage as measured by terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) (Zhu et al., 2006) as well as partially attenuating against excess reactive oxygen and nitrogen species (Tanaka et al., 1999). Research from our own laboratory has demonstrated that stimulation of astrocytic β 2-adrenoceptors promotes the release of trophic factors including BDNF, NGF- β , FGF-2, glial-derived neurotrophic factor (GDNF), and Interleukin 6 (IL-6), which lead to an increase in the growth of primary cortical neurons *in vitro* (Day et al., 2014). These results indicate that astrocytes can be primed into a trophic phenotype by increasing noradrenergic tone, and highlights the potential for targeting astrocytes as a strategy to encourage neuronal remodelling following CNS injury.

1.14.2 The role of astrocytes in synaptogenesis

An increasing body of evidence demonstrates that astrocytes play a crucial role in synapse formation and maturation (Ullian et al., 2001, Ullian et al., 2004, Christopherson et al., 2005, Molofsky et al., 2012, Allen, 2013). Interestingly, a temporal correlation exists between glial differentiation and the formation of synapses in the developing CNS, and astrocytic dysfunctions

are noted as a prominent feature of many neurodevelopmental disorders which are linked to synaptic deficits, most notably autism and schizophrenia (Clarke and Barres, 2013). Astrocytes have been shown to profoundly increase the number and efficacy of synapses *in vitro*, and are known to be crucial for the formation of fully functional synapses as measured by patch clamp technique and immunostaining for pre- and post-synaptic marker co-localisation (Ullian et al., 2004). Early research observed that retinal ganglion cells (RGCs) cultured in the absence of glia formed synapses which were largely devoid of spontaneous electrical activity, while RGCs grown in a glial co-culture displayed a 70-fold and 5-fold increase in the frequency and amplitude of spontaneous post-synaptic currents, respectively (Pfrieger and Barres, 1997). Research by Nädler et al. (2001) also found that cultured RGCs formed very few and inefficient autapses (a synapse made between an axon and dendrite of the same neuron) when cultured in the absence of glial cells. These authors report that the addition of glial-derived CM was capable of increasing the number of functional autapses by up to 10-fold as well as promoting the formation of new autapses. This research indicates that soluble factors derived from glia are capable of promoting synapse formation and maturation. In further support of this, Ullian et al. (2001) demonstrated that few synapses formed between purified RGCs in culture in the absence of astrocytes, whereas the presence of astrocytes increased the number of synapses by nearly 10-fold.

This research suggests that astrocytes actively secrete molecules which promote synaptogenesis, and these molecules were later found to include hevin (also known as SPARCII) (Allen, 2013), cholesterol complexed to apolipoprotein-E containing lipoproteins (Mauch et al., 2001) as well as thrombospondins (TSPs) - a family of five homologous, matrix-associated proteins expressed in the brain exclusively during development and injury (Christopherson et al., 2005). TSPs have been shown to be sufficient to induce synaptogenesis *in vitro*, and TSP-1/2 knockout animals have a significantly reduced number of synapses, demonstrating the importance of astrocytes in synapse formation *in vivo* (Christopherson et al., 2005). Astrocytes have also been shown to mediate synaptic strength through the production of glial-derived TNF α which significantly increased post-synaptic efficacy by increasing GluR1 AMPA-R subunit expression at the post-synaptic membrane of excitatory synapses (Beattie et al., 2002). Glial-derived TNF α has also been reported to play a role in synaptic scaling (Stellwagen and Malenka, 2006), further implicating astrocyte-derived factors in neuronal circuitry refinement and synaptic plasticity.

1.15 L-alpha-aminoadipic acid (L-AAA) as a tool to study astrocytic dysfunction in disease pathophysiology

L-alpha-aminoadipic acid (L-AAA) is a glutamate analogue which has been shown to be selectively toxic to astrocytes (Brown and Kretschmar, 1998, Nishimura et al., 2000).

Aminoadipic acid (AAA) occurs naturally in the brain at micromolar concentrations (~30 μM) and is an intermediate in lysine metabolism (Wu et al., 1995). *In vitro* studies on cultured rat astrocytes have shown that L-AAA is uptaken into astrocytes through the Na^+ -dependent glutamate transporters (i.e. GLAST and GLT-1) and also partly through a Na^+ - independent process (i.e. through chloride-dependent glutamate/cysteine antiporter) (Tsai et al., 1996, Brown and Kretzschmar, 1998). Its mechanism of selective toxicity to astrocytes is not fully elucidated but is thought to involve inhibition of the glutamate transporters in addition to competitive inhibition of glutamine synthetase, the astrocytic enzyme associated with glutamate metabolism, and γ -glutamylcysteine synthetase, an enzyme involved in the synthesis of the important antioxidant glutathione (Olney et al., 1980, McBean, 1994). L-AAA-mediated astrocyte toxicity may therefore involve glutamate accumulation in astrocytes and/or oxidative stress. More recent research also indicates that 24 h exposure of cultured rat astrocytes to L-AAA (1 mM) leads to a reduction in protein synthesis as measured by [^{35}S]-methionine labelling (Nishimura et al., 2000). Endogenous AAA is degraded by transamination to α -oxoadipic acid by the enzyme AAA aminotransferase [also called kynurenine aminotransferase II (KATII)]. This enzyme is found predominantly in non-neuronal cells and is also responsible for catalysis of kynurenine to the neuroprotective excitatory amino acid receptor antagonist kynurenic acid (Tsai et al., 1996, Han et al., 2009). Interestingly, L-AAA has been reported to reduce kynurenic acid levels in the hippocampus of rats (Wu et al., 1995), an observation which may contribute to the weak excitatory neuronal responses observed with L-AAA *in vitro* (MacDonald and Wojtowicz, 1982).

Since L-AAA is regarded as an astrocyte specific toxin, it represents an important tool to investigate the specific functions of astrocytes on disease pathology *in vivo*. Of particular interest, L-AAA has increasingly been used in recent years by several research groups to investigate the role of astrocytes in depression (Banasr and Duman, 2008, Domin et al., 2014, Lima et al., 2014). In this regard, L-AAA microinjection into the PFC of rats is sufficient to induce depression-like symptoms - similar to the behavioural consequences observed following chronic unpredictable stress (CUS) paradigms - indicating a role of astrocytes in depression pathophysiology. Importantly, ablation of neurons by the neurotoxin ibotenate had no effect on these behavioural tests - indicating astrocytes in particular as sufficient to induce depression-like behaviour. L-AAA-induced depressive symptoms can be attenuated using conventional antidepressants, thus supporting the predictive validity of this toxin as an animal model of depression (Domin et al., 2014). Interestingly, additional studies using this toxin have reported that L-AAA-induced astrocytic ablation in the medial PFC results in a progressive neuronal degeneration in the lesion site that is accompanied by dendritic atrophy in surviving neurons, in addition to behavioural alterations including alterations in attentional set shifting. These results suggest that an astroglial

dysfunction leads to an indirect neuronal loss and neuronal atrophy, which may in turn contribute to the cognitive deficits exhibited by these animals (Lima et al., 2014).

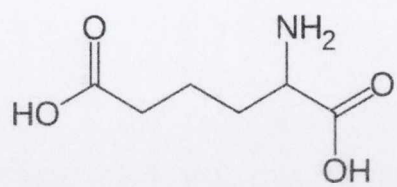


Figure 1.13: Structure of L-alpha aminoadipic acid (L-AAA). L-AAA is a glutamate analogue that differs from glutamate by a single methylene group. It has been shown to exhibit specific astrocytic toxicity by mechanisms involving astrocyte uptake through glutamate transporters prior to intracellular accumulation and protein synthesis inhibition.

1.16 Hypotheses and Aims

Inhibition of the NMDA-R/NO signalling pathway through the use of CPPs and small molecular weight inhibitor have been shown to provide protective effects in animal models of pain, ischaemia, depression and anxiety (Florio et al., 2009, Zhou et al., 2010, Doucet et al., 2013, Zhu et al., 2014), and several reports suggest that these neuroprotective effects may be mediated through the promotion of neurotrophic effects (Li et al., 2010, Zhou et al., 2015). Since neuronal atrophy and changes in neurite morphology are considered an early hallmark of glutamate-induced neuronal dysfunction and precede cell death, we hypothesise that disruption of elements of NMDA-R and nNOS signalling will provide a strategy to counteract neuronal atrophy and encourage neurite outgrowth. In addition, considering the important role of astrocytes in neuronal growth, we hypothesise that astrocytic dysfunctions may increase the susceptibility of neurons to atrophy. In order to investigate these hypotheses, the current research employed the use of an *in vitro* model composed of primary cortical neurons to study the effects of NMDA-R and NO modulation on neurite outgrowth responses. The specific aims of this research were:

1. To examine the effects of glutamate, NMDA and increased NO production on neurite outgrowth responses. The impact of these compounds on the co-localisation of pre- and post-synaptic protein expression as a measure of synapse number was also examined. Following on from this, the aim was to assess the ability of NMDA-R antagonism and nNOS inhibition, as well as small-molecule inhibition of the PSD-95/nNOS interface, using IC87201 and ZL006, to protect against NMDA-induced neuronal atrophy.
2. To determine the role for the NOS1AP/nNOS interaction in neurite outgrowth and synaptic protein co-localisation by testing the ability of the peptide inhibitor L-TAT-GESV to protect against NMDA-induced atrophy. Since the detrimental effects of NMDA has been linked to the activation of downstream JNK and p38 MAPK signalling, we also assessed the impact of JNK and p38 MAPK inhibition on NMDA-induced neurite atrophy.
3. To characterise the effects of the astrocyte toxin, L-AAA, *in vitro* and to determine the effects of soluble mediators derived from L-AAA-treated astrocytes on neurite outgrowth and synaptic protein co-localisation. Since we hypothesise a putative role for NMDA-R/NO signalling inhibition in the promotion of neurotrophic effects, the final aim was to determine whether NMDA-R antagonism and small-molecule inhibition for the PSD-95/nNOS interaction can protect against L-AAA-mediated neurite atrophy.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell culture products

Absolute ethanol	Hazmat, TCD
Antimycin A	Sigma-Aldrich, Ireland
B-27 supplement	Invitrogen, Ireland
Biocidal ZF™	WAK-Chemie Medical, Germany
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone	Sigma-Aldrich, Ireland
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Ireland
Dulbecco's modified Eagle medium (DMEM)	Sigma-Aldrich, Ireland
Dulbecco's phosphate buffered saline (DPBS) (1x)	Invitrogen, Ireland
DPBS (10x)	Invitrogen, Ireland
Fetal bovine serum (FBS) heat inactivated	Biosera, UK
Fungizone	Invitrogen, Ireland
Glucose	Sigma-Aldrich, Ireland
Glutamax	Invitrogen, Ireland
Glycine	Fisher Scientific, Ireland
L-glutamine	Sigma-Aldrich, Ireland
Methanol	Fisher Scientific, Ireland
Neurobasal A medium	Invitrogen, Ireland
Normal goat serum	Sigma-Aldrich, Ireland
Oligomycin	Calbiochem
Penicillin/streptomycin	Sigma-Aldrich
Phosphate buffered saline (PBS)	Invitrogen, Ireland
Poly-D-lysine hydrobromide	Sigma-Aldrich, Ireland

Rezazurin (Alamar Blue Assay)	Sigma-Aldrich, Ireland
Rotenone	Sigma-Aldrich, Ireland
Sodium hydroxide (NaOH)	Sigma-Aldrich, Ireland
Trypan blue solution (0.4%)	Sigma-Aldrich, Ireland
Trypsin-EDTA solution (0.25%)	Sigma-Aldrich, Ireland
Un-buffered DMEM	Seahorse, Biosciences
Vectashield mounting medium with DAPI	Vector Laboratories, UK
Virkon	Antec International, USA
Wistar rats	Comparative Medicine, TCD
XF Calibrant	Seahorse, Biosciences

2.1.2 General consumables

6-, 24- and 96-well plates	Sarstedt, Ireland
Cell culture flasks (T-75 cm ²)	Corning, Ireland
Cell scrapers	Sarstedt, Ireland
Cell strainers (40 µm), BD Falcon	VWR, Ireland
Falcon tubes (15 ml, 50 ml), sterile	Sarstedt, Ireland
Filter units (0.22 µm), single-use (Millex)	Millipore, Ireland
Glass coverslips (13 mm, 35 mm)	VWR, Ireland
Haemocytometer	VWR, Ireland
Microscope slides 76 mm x 26 mm	Fisher Scientific, Ireland
Microtubes (0.5 ml, 1.5 ml, 2 ml)	Sarstedt, Ireland
Parafilm	Fisher Scientific, Ireland
Pasteur pipettes (3.5 ml)	Sarstedt, Ireland

Pipette tips (200 µl, 1000 µl)	Sarstedt, Ireland
Pipettes (5 ml, 10 ml, 25 ml), sterile	Sarstedt, Ireland
Petri dishes (90 mm)	Fisher Scientific, Ireland
Scalpels, disposable, sterile (Swann-Morton)	Fisher Scientific, Ireland
Syringes (20ml, 50ml)	Fisher Scientific, Ireland
XF24-well cell culture plate	Seahorse, Biosciences

2.1.3 General equipment

Autoclave	Sanyo, Ireland
Biosafety cabinet	ADS Laminare, France
Centrifuge: Legend RT+	Sorvall, Ireland
Gyro rocker	Stuart, Ireland
Haemocytometer	VWR, Ireland
Incubator (Innova CO-48)	New Brunswick, Ireland
Microscope: CKX41	Olympus, UK
Microscope: Axio Imager Z1	Carl Zeiss, UK
Nanodrop® ND1000 Spectrophotometer	Thermo Fisher Scientific
pH meter	Mettler-Toledo, Ireland
Vortex	Ika, Ireland
Water bath	Grant, Ireland
Plate reader	Biotek
XF24 Analyzer	Seahorse, Biosciences

2.1.4 Experimental treatments

BIRB796 (Doramapimod)	University of Turku, Finland
Glycine	Sigma-Aldrich, Ireland
Ketamine (hydrochloride) (Vetalar V®)	Pfizer, Ireland

D-arginine	Sigma-Aldrich, Ireland
D-JNKI-1	University of Turku, Finland
L-arginine	Sigma-Aldrich, Ireland
L-2-Aminoadipic Acid (L-AAA)	Sigma-Aldrich, Ireland
L-glutamic acid	Sigma-Aldrich, Ireland
L-TAT	University of Turku, Finland
L-TAT-D1	University of Turku, Finland
L-TAT-GASA	University of Turku, Finland
L-TAT-GESV	University of Turku, Finland
MK-801	Tocris Bioscience, UK
N-methyl-D-aspartic acid (NMDA)	Sigma-Aldrich, Ireland
SB203580	University of Turku, Finland
Sodium Nitroprusside (SNP) dihydrate	Sigma-Aldrich, Ireland
1-[2-(trifluoromethyl)phenyl] imidazole (TRIM)	Alfa Aesar, UK
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	Sigma-Aldrich, Ireland
2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl)- 4,6-dichlorophenol (IC87201)	TopChem, Ireland
4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxy benzoic acid (ZL006)	TopChem, Ireland
2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline- 7-sulfonamide (NBQX) (disodium salt)	Tocris, UK

2.1.5 Molecular reagents

Biosphere filter tips (10, 20, 100, 200, 1000µl)	Sarstedt Ltd., Ireland
β-Mercaptoethanol	Sigma Aldrich, Ireland
High capacity cDNA archive kit	Applied Biosystems, UK
Microamp 96-well Reaction plate	Applied Biosystems, UK
Molecular grade ethanol (200 proof)	Sigma-Aldrich, Ireland
Nucleospin® RNA II Isolation Kit	Macherney-Nagel, Germany
Optical adhesive covers	Applied Biosystems, UK
RNase-free microtubes	Ambion Inc., USA
RNaseZap® wipes	Ambion Inc., USA
TaqMan® Universal PCR Master Mix	Applied Biosystems, UK
TaqMan® Gene Expression Assays	Applied Biosystems, UK

2.1.6 Fluorescent Immunocytochemistry

Table 2.1: List of Antibodies used for Fluorescent Immunocytochemistry

Name	Host species	Company	Catalog number	IF dilution
Primary antibodies:				
β-III tubulin	Mouse	Promega, Ireland	G7121	1:1000
GFAP	Rabbit	Dako, Ireland	Z033429-2	1:1000
CD11b	Mouse	Millipore, Ireland	CBL1313	1:1000
Synaptophysin	Rabbit	Thermo Scientific	PA1-1043	1:500
Post-synaptic density-95	Mouse	Fischer Scientific	11508772	1:500
Secondary antibodies:				
Alexa Fluor 488 anti-mouse	Goat	Invitrogen, Ireland	A11029	1:2000
AlexaFluor 594 anti-rabbit	Goat	Invitrogen, Ireland	A11003	1:1000
Alexa Fluor 647 anti-rabbit	Goat	Invitrogen, Ireland	A20991	1:2000

2.2 Methods

2.2.1 Aseptic Technique

Aseptic techniques were utilised during all cell culture work and also in the preparation of cell culture reagents. These techniques are necessary in order to maintain a sterile environment free from fungal, bacterial and viral infections which have the potential to alter normal cellular functions. Pipette tips and distilled H₂O (dH₂O) were sterilised by autoclaving at 121°C for 30-60 min prior to use. Sterile plastic ware was also used and dissection equipment was cleaned with Virkon. All cell culture work was carried out in a laminar flow hood. This allows only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The interior of the hood was wiped down with 70% ethanol (EtOH) before and after use. The hood surface was also exposed to ultraviolet (UV) light for 15-30 min after use. Any items taken into the flow hood were lightly sprayed with 70% EtOH to prevent introduction of any pathogens to the hood work area. Disposable latex gloves were worn at all times while working in the laminar flow hood and were sprayed with 70% EtOH before use. Gloves were changed regularly during cell culture work. Cells were maintained in an incubator at 37°C in a 5% CO₂ humidified atmosphere. Both the incubator and laminar flow hood were regularly cleaned with Biocidal to maintain a sterile environment.

2.2.2 Preparation of culture media

Primary glial cultures: Dulbecco's modified Eagle's medium (DMEM) Ham's F12 was used for the maintenance of primary glial cells and was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.1% fungizone (referred to as complete DMEM (cDMEM) hereafter). Briefly, 50 ml of FBS, 5 ml of penicillin-streptomycin and 500 µl of fungizone were filter-sterilised using a 0.2 µm syringe filter and added to a 500 ml bottle of DMEM. The cDMEM was stored by refrigeration at 4°C for further use.

Primary neuronal cultures: Neurobasal A medium (NBM) was used for maintenance of primary neuronal cultures and was supplemented with 1% glutamax, 0.1% fungizone and 1% penicillin-streptomycin (referred to as complete NBM (cNBM) hereafter). Briefly, 5 ml of penicillin-streptomycin, 500 µl of fungizone and 5 ml of glutamax, were filter-sterilised using a 0.2 µm syringe filter and added to a 500 ml bottle of NBM. The cNBM was stored by refrigeration at 4°C for further use. When required, a working solution was prepared by adding 500 µl of 100 X B-27 supplement to 50 ml of cNBM.

Phosphate buffered saline (PBS): A working solution of 1 X PBS was prepared by adding 5 ml Dulbecco's sterile 10 X PBS to 45 ml dH₂O.

Alamar Blue (Resazurin): A 440 μM stock solution of alamar blue was prepared by dissolving 25 mg resazurin ($\text{C}_{12}\text{H}_6\text{NNaO}_4$, Molecular weight 251.2 g/mol) in 226.2 ml dH_2O . Stock solution was stored in a dark bottle for future use.

2.2.3 Preparation of agents for treating cell cultures

Once prepared, all stock solutions were filter-sterilised using a 0.2 μm syringe filter and frozen at -20°C in aliquots for future use, except for sodium nitroprusside (SNP) which was prepared freshly for each use. Stock solutions were diluted to working concentrations in appropriate pre-warmed media prior to use.

Table 2.2: Table of compounds for cell treatments

Compound name	Molecular weight (g/mol)	Stock Concentration	Dissolved in:
BIRB796	527.66	4 mM	DMSO
Glutamate	147.13	10 mM	cNBM
Glycine	75.06	10 mM	cNBM
IC87201	309.15	20 mM	DMSO
Ketamine	237.725	1 mM	cNBM
L-AAA	161.16	50 mM	cNBM
L-arginine	174.2	10 mM	cNBM
MK-801	337.37	1 mM	cNBM
NBQX	380.24	1 mM	cNBM
NMDA	147.13	10 mM	cNBM
ODQ	187.15	10 mM	DMSO
SB203580	377.4	10 mM	DMSO
SNP	297.95	10 mM	cNBM
TRIM	212.2	10 mM	cNBM
ZL006	328.15	1 mM	cNBM

Table 2.3: Table of TAT-fused peptides for cell treatments

Peptide name	Sequence	Mr (theoretical)	Purity (%)	Stock Conc.	Dissolved in
L-TAT	NH2-GRKKRRQRRR-COOH	1396.68	97.43	10 mM	dH ₂ O
L-TAT-D1	Sequence information has not been disclosed for this peptide	2695	95	1 mM	dH ₂ O
L-TAT-GASA	NH2-GRKKRRQRRRYAGQWGAS A-COOH	2288.63	97.81	10 mM	dH ₂ O
L-TAT-GESV	NH2-GRKKRRQRRRYAGQWGES V-COOH	2374.72	96.93	10 mM	dH ₂ O
D-JNK1-1	DQSRPVQPFLNLTPRKPRPP RRRQRRKKR)G-NH2.	3822.44	97.33	10 mM	dH ₂ O

2.2.4 Preparation and culture of primary glial cells

2.2.4.1 Preparation of primary cortical mixed glial cells

Primary cortical glial cells were prepared as described previously (Day et al., 2014). Specifically, cortical brain tissue was dissected from three postnatal day 2 or 3 (P 2-3) Wistar rat pups in the laminar flow hood under sterile conditions. Pups were decapitated using a large scissors, and a smaller scissors was then used to expose the brain by cutting along the midline, firstly through the skin and then through the skull. A straight forceps was used to remove the meninges and a curved forceps was used to carefully remove the brain, which was placed on a Petri Dish with a drop of pre-warmed, cDMEM. The cortical tissue from both hemispheres was subsequently dissociated and the rest of the brain was discarded. The cortices from each of the three rat pups were then pooled together and cross-chopped using a sterile scalpel. Any obvious blood vessels were removed at this point. The finely chopped cortical tissue was then placed in 6 ml pre-warmed cDMEM and placed in the incubator for 20 min at 37°C. After this incubation period, the tissue was triturated until homogenous, passed through a sterile mesh filter (40 µm) and centrifuged at 2000 rpm for 3 min at 20°C. The supernatant was discarded and the pellet was re-suspended in 1 ml pre-warmed cDMEM by gently triturating until a homogenous cellular suspension was obtained. The cell suspension was then diluted with 15 ml pre-warmed cDMEM and 5 ml of this suspension was added to each of 3 x T75 flasks. The flasks were placed into an incubator at 37°C, 5% CO₂ for 2 h to allow adherence to the bottom of the flask. After 2 h, the flasks were flooded with 10 ml cDMEM. The day of plating was referred to as days *in vitro* 0 (DIV 0). Media was replaced on DIV 3 and DIV 7 or DIV 8 by pouring off the old media and adding 15 ml fresh, pre-warmed cDMEM to the flasks by gently pipetting the cDMEM to the sides of the flask in order to

prevent disturbing the cells. Glial cells were confluent and were separated at approximately DIV 10.

2.2.4.2 Mixed glial isolation and re-plating

After primary mixed glial cells reached confluency (DIV 10 approximately), a cell scraper was used to remove mixed glial cells from the flasks. The resulting suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was discarded and the pellet re-suspended in 1 ml pre-warmed cDMEM. The cell suspension was counted by the trypan blue exclusion method which works on the basis that live cells possess intact cellular membranes which have the ability to exclude trypan blue dye whereas dead cells do not. Trypan blue was used at a dilution of 1:10 with cDMEM and viable cells (cells capable of excluding Trypan blue) were counted using a glass haemocytometer under a light microscope. For fluorescent immunocytochemistry assays, the cell suspension was diluted in pre-warmed cDMEM to a density of 1×10^5 cells/ml and 75 μ l of this dilution was gently pipetted onto uncoated, sterile glass coverslips in 24-well plates for 2 h to allow adherence of the cells to the coverslip, before the addition of 300 μ l cDMEM. For viability assays, the cells were re-plated in the same way with the exception that no coverslips were used in the 24 well plates. After plating, the cells were left for at least 2 days until confluent (until approximately DIV 12/13) in the 24-well plates before fixation for staining.

2.2.4.3 Astrocyte isolation and re-plating

To obtain isolated cultures of astrocytes, primary mixed glial cells were cultured from three postnatal day 2 or 3 (P 2-3) Wistar rat pups as described above. After primary mixed glial cells reached confluency (DIV 10 approximately), the flasks were wrapped in parafilm and agitated on an orbital shaker for 2 h at 110 rpm to remove the non-adherent microglia and oligodendrocytes cells from the astrocytic monolayer. The flasks were then tapped firmly 10 times in the laminar flow hood to encourage the microglia and oligodendrocytes to fully detach from the surface of the flasks. This media was poured off and discarded. The remaining astrocytic monolayer was gently bathed in 5 ml pre-warmed cDMEM and collected from the flasks using a cell scraper. The resulting suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was discarded and the pellet re-suspended in 1 ml pre-warmed cDMEM. Astrocytes were diluted with trypan blue at a dilution of 1:10 with cDMEM and counted using a glass haemocytometer under a light microscope. For fluorescent immunocytochemistry to confirm astrocyte purity using the astrocyte specific marker, glial fibrillary acidic protein (GFAP), the cell suspension was diluted in pre-warmed cDMEM to a density of 1×10^5 cells/ml and 75 μ l of this dilution was gently pipetted onto uncoated, sterile glass coverslips in 24-well plates for 2 h to allow adherence of the cells to the coverslips, before the addition of 300 μ l cDMEM. For PCR experiments and viability assays, astrocytes were re-plated in the same way with the exception that no coverslips were used in the

24 well plates. For conditioned media studies, the astrocytic cell suspension was diluted in pre-warmed cDMEM to a density of 5×10^5 cells/ml and 1 ml of this dilution was gently pipetted onto uncoated 6-well plates. After plating, the astrocytes were left for at least 2 days in the 6-well plates before treatment for conditioned media studies.

2.2.4.4 Microglial isolation and re-plating

To obtain isolated cultures of microglia, primary mixed glial cells were cultured from three postnatal day 2 or 3 (P2-3) Wistar rat pups as described above, except that the cultures for microglia were treated with Granulocyte-macrophage colony-stimulating factor GM-CSF (10 ng/ml) and macrophage colony-stimulating factor (M-CSF) (10 ng/ml) one day after culturing (DIV 1) to encourage microglial proliferation. Media was changed every 3-4 days and was supplemented with fresh GM-CSF (10 ng/ml) and M-CSF (10 ng/ml). After these cells reached confluency (DIV 10 approximately), the flasks were wrapped in parafilm and agitated on an orbital shaker for 2 h at 110 rpm. The flasks were brought back into the laminar flow and tapped 10 times to encourage microglia to fully detach. This resultant suspension containing detached microglia was collected and centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was discarded and the pellet re-suspended in 1 ml cDMEM and counted by the trypan blue method. For fluorescent immunocytochemistry to confirm microglial purity using the microglial specific marker CD11b, the cell suspension was diluted in pre-warmed cDMEM to a density of 1×10^5 cells/ml and 75 μ l of this dilution was gently pipetted onto uncoated, sterile glass coverslips in 24-well plates for at least 3 h to allow adherence of the cells to the coverslip, before the addition of 300 μ l cDMEM. For viability assays, microglia were re-plated in the same way with the exception that no coverslips were used in the 24 well plates. After plating, the microglia were left for at least 2 days until confluent (until approximately DIV 12) in the 24-well plates before fixation for staining.

2.2.4.5 Glial cell treatments

For viability assays and PCR experiments, the mixed glia, purified astrocytes or purified microglia were treated on approximately DIV 13 with various agents diluted in pre-warmed cDMEM for the appropriate length of time. A volume of 300 μ l was added per well into 24-well plates.

2.2.4.6 Astrocyte treatments for conditioned media (CM) studies

To obtain astrocyte conditioned media, cells were grown in 6-well plates and treated with a volume of 1 ml of compounds (L-AAA or glutamate) diluted in pre-warmed cNBM for the appropriate length of time (24 h or 48 h). After this time, the resulting conditioned media from each well of a particular treatment was collected with a Pasteur pipette and pooled together in a

15 ml falcon tube followed by centrifugation at 2,000 rpm for 3 min at 20°C to remove any cellular debris. The resulting supernatant was collected and filter sterilised using a 0.2 µm syringe filter. The conditioned media was then stored at -20°C for further use.

2.2.5 Preparation and culture of primary neurons

2.2.5.1 Preparation of coverslips

Poly-D-lysine was prepared by reconstituting 5 mg with 50 ml sterile H₂O to make a stock concentration of 0.1 mg/ml. This solution was then filter-sterilised using a 0.2 µm syringe filter and frozen at -20°C in 5 ml aliquots until further use. When required, the poly-D-lysine aliquot was further diluted by addition of 5 ml sterile dH₂O to a working concentration of 50 µg/ml. Glass coverslips were sterilised by placing in an oven at 200°C for at least 6 h, and were then placed into 24-well plates and further sterilised by exposing them under UV light for 1 h. Poly-D-lysine (100 µl) was subsequently added into the centre of each coverslip and incubated for 1 h at 37°C. Following this, the poly-D-lysine solution was removed and kept at 4°C to be re-used up to three times. The coverslips were then rinsed twice with sterile dH₂O. The plates were left to fully dry for at least 2 h until further use.

2.2.5.2 Preparation of primary cortical neuronal cultures

Neuronal cultures were prepared from postnatal day 1 (P1) Wistar rat pups as described previously (Day et al. 2014; McNamee et al. 2010). Briefly, the cortex was dissected using the same technique as outlined for glial cell preparations and the tissue was finely cross-chopped in a drop of pre-warmed cNBm. The tissue was then transferred into trypsin-EDTA (5 ml), and incubated for 2 min at 37°C. cDMEM (5 ml) (which contains FBS and therefore deactivates the trypsin) was then added and the tissue was triturated, followed by centrifugation at 2,000 rpm for 3 min at 20°C. The resultant supernatant was discarded and the pellet was re-suspended in cDMEM (5 ml). This cell suspension was triturated until no visible clumps remained, passed through a sterile mesh filter (40 µm) and centrifuged at 2,000 rpm for 3 min at 20°C. The supernatant was discarded and the pellet was re-suspended in 1 ml of pre-warmed cNBm + B27 by gently triturating until a homogenous cellular suspension was obtained.

2.2.5.3 Neuronal cell counting and plating

The neuronal cell suspension was counted by the trypan blue exclusion method. Cells were diluted with trypan blue at a dilution of 1:100 and counted using a glass haemocytometer under a light microscope. For Sholl analysis and synaptic protein co-localisation experiments, the cell suspension was diluted to 3×10^5 cells/ml and 75 µl of this dilution was gently pipetted onto the centre of a poly-D-lysine coated coverslip in 24-well plates. The plates were left in an incubator at 37°C, 5% CO₂ for 2 h to allow adherence of the cells to the coverslip, followed by the addition of

300 µl cNBM. The day of plating was referred to as DIV 0. For Sholl analysis experiments, neurons were treated with compounds at DIV 3 and fixed for subsequent immunocytochemistry and Sholl analysis at DIV 4. Neurons for synaptic protein co-localisation studies were maintained in culture by replacing one third of the media in each well (100 µl) with fresh pre-warmed cNBM after 6 days of culture (DIV 6) and then every subsequent 3-4 days. These neurons were treated with compounds at DIV 19 and fixed for subsequent immunocytochemistry and imaging at DIV 20.

2.2.6 RNA Analysis

2.2.6.1 Harvesting cells for mRNA analysis

Prior to cell harvesting, all equipment was wiped with RNase Zap wipes in order to prevent RNA contamination. Following cell culture treatment, supernatants were aspirated from wells and 100 µl RA1 lysis buffer (supplied in Total RNA isolation kit; 350 µl) containing 1% β-mercaptoethanol was pipetted into each of the wells. Cells were scraped from the base of the wells using a 1000 µl filtered pipette tip and the lysate was transferred to a 2 ml RNase-free microtube. Pipette tips were changed between treatment groups in order to prevent cross contamination. Lysates were stored at -80°C for subsequent RNA extraction.

2.2.6.2 RNA Extraction

RNA was isolated from samples using a Nucleospin® RNA II extraction kit as per the manufacturer's instructions. Cell lysates were added to Nucleospin® filter columns in a collecting tube and centrifuged for 1 min at 13,000 rpm. The filter column was discarded and 350 µl of 70% ethanol was added to the lysate and mixed by pipetting up and down approximately five times. The mixture was then applied to different Nucleospin® Filter columns and centrifuged for 30 s at 13,000 rpm in order to let the RNA bind to the silica column. This column was then placed in a new collecting tube (2 ml) and 350 µl of membrane desalting buffer (MDB) was added to the column. This was centrifuged for 1 min at 13,000 rpm. A DNase reaction mixture was prepared in a sterile 1.5 ml microcentrifuge, which contained 10 µl of reconstituted rDNase to 90 µl rDNase reaction buffer. 95 µl of this mixture was added directly onto the centre of the silica membrane of the column and was incubated at room temperature for 15 min. Following this a series of washes were carried out to digest any contaminating DNA on the membrane. Firstly, 200 µl of Buffer RA2 was added to the column to inactivate the rDNase followed by centrifugation for 30 s at 13,000 rpm. The column was placed in a new tube and the membrane was dried by the addition of 600 µl RA3 buffer (to which 50 ml of 200-proof ethanol had been added to the 25 ml RA3 buffer concentrate) and centrifuged at 11000 x g for 30 s. The third and final wash entailed adding 250 µl of Buffer RA3 to the column and this was centrifuged at 13,000 rpm for 2 min in order to dry the membrane completely. The column was then placed into a nuclease-free collection tube and

the flow-through discarded. Finally highly pure RNA was eluted in 60 µl of RNase free H₂O and centrifuged at 13,000 rpm for 1 min. The pure RNA was then frozen at -80°C until RNA quantification and equalisation.

2.2.6.3 RNA Quantification and Equalisation

Total RNA concentrations were measured using a Nanodrop® ND1000 Spectrophotometer. RNase-free H₂O was used to blank the machine. Purity was demonstrated by the A_{260/280} ratio where a ratio of 2 indicates highly pure RNA. RNA samples were equalised using RNase-free H₂O to the lowest concentration of RNA detected.

2.2.6.4 cDNA Synthesis

Complementary DNA (cDNA) was reverse transcribed from the equalised RNA using ABI High Capacity cDNA archive kit according to the manufacturer's protocol. Briefly, an equal volume of RNA was mixed with cDNA Mastermix (containing RT Buffer (Reverse Transcriptase), dNTPs, random primers, multiscribe RT and RNase-free H₂O). The samples were mixed, briefly centrifuged and placed into a Thermal Cycler (PTC-200 Peltier Thermal Cycler DNA Engine) on the ABI-RT programme. The samples were kept at 25°C for 10 min and then at 37°C for 120 min. Upon completion of the programme, the samples were removed and frozen at -20°C until polymerase chain reaction (PCR) analysis.

2.2.6.5 Real-time Polymerase Chain Reaction (RT-PCR)

Assessment of target genes was performed as described previously (Boyle and Connor, 2007, Day et al., 2014) and was carried out using Taqman® Gene Expression Assays, containing specific target primers and a FAM® dye labelled MGB (minor groove binding) target probe. β-actin was used to normalise gene expression between samples, and was quantified using a β-actin endogenous control gene expression assay containing specific primers, and a VIC-labelled NGB probe for rat β-actin. cDNA was diluted 1:4 with RNase-free H₂O and a 4 µl volume of the diluted cDNA samples were pipetted into a PCR plate, along with 0.5 µl of target primer, 0.5 µl of β-actin primer, and 5 µl of Taqman® Universal PCR Master Mix. Electronic pipettes (EDP3 20-200µl, 2-20µl, and 10-100µl) were used in order to ensure accuracy. RT-PCR was carried out using StepOne software. Samples were assayed in one run (40 cycles), composed of 2 stages:-

- Holding stage; 50°C for 2 min, and 95°C for 10 s
- Cycling stage; 95°C for 1 s, and 60°C for 1 min.

Target gene expression was assessed relative to β-actin, the endogenous control. The CT method (Applied Biosystems RQ software, UK) was utilised in the analysis of all RT-PCR data. The basis of this method is to assess relative gene expression by comparing gene expression of treated

samples to a normal or untreated sample; this allows assessment of the fold-difference between control and treated samples. Briefly, a threshold for fluorescence is set, against which CT is measured. To accurately assess differences between gene expression the threshold is set when the PCR reaction is in the exponential phase, when the PCR reaction is optimal or 100% efficient. Thus, samples with low CT readings demonstrate high fluorescence, indicating greater amplification and hence, greater gene expression. When a PCR is 100% efficient a one-cycle difference between samples means a 2-fold difference in copy number (2^2), similarly a 5-fold difference is a 32-fold difference (2^5).

Table 2.4: List of Taqman gene expression assays used

Target:	Primer Code:
β-actin (VIC)	4352340E
Caspase-3	Rn00563902_m1
GFAP	Rn00566603_m1
GLAST (slcla3)	Rn00570130_m1
GLT1 (slcla2)	Rn00568080_m1
iNOS (NOS2)	Rn00561646_m1
KAT II (AADAT)	Rn00567882_m1
S100B	Rn00566139_m1
TNFα	Rn00562055_m1

2.2.7 Cell Viability Resazurin (Alamar Blue®) Assay

The Alamar blue assay is a metabolic assay used for assessing the ability of living cells to convert a redox dye (resazurin, $C_{12}H_6NNaO_4$) into a fluorescent end product (resorufin) (O'Brien et al., 2000, Rampersad, 2012). Viable cells can reduce resazurin into resorufin via mitochondrial metabolism. The bioreduction of the dye reduces the amount of the oxidized form (blue) and concomitantly increases the fluorescent intermediate (red). A 440 μM solution of Alamar Blue dye was prepared by dissolving 25 mg resazurin in 226 ml dH₂O. This stock solution was then stored in a dark bottle for future use. A working concentration of 44 μM resazurin was prepared by diluting resazurin 1:10 in the appropriate pre-warmed fresh media (cNBM for neuronal assays and cDMEM for glial assays). Media was removed from the cultures and the resazurin solution was then added. Resazurin solution was also added to empty wells with no cells as a blank to use for comparison to treated cells. For cells in 24-well plates, a total volume of 300 μl was added per well (30 μl Alamar Blue, 270 μl media). For cells plated in 6-well plates, a total volume of 600 μl

was added per well (60µl Alamar Blue, 540 µl media). Plates were incubated for 2-4 h at 37°C in a humidified, 5% CO₂ atmosphere. Following a colour change, aliquots of supernatants (100 µl) were transferred in triplicate to a 96-well flat-bottomed plate. Absorbance was read at 600 nm using a microtitre plate reader. The percentage of cell viability was calculated according to the following equation:

$$\% \text{ Cell Viability} = ((Ab - As)/(Ab - Ac)) \times 100$$

Where Ab = Average absorbance of wells containing media only (Blanks),

Ac = Average absorbance of wells containing cells without treatments (Controls)

As = Absorbance of a particular sample

2.2.8 Measurement of oxygen consumption rate (OCR) using Extracellular Flux Analyser

The oxygen consumption rate (OCR) was assessed using an Extracellular Flux (XF) Analyser (Seahorse Biosciences). XF analysis is a non-invasive procedure that allows for the determination of cellular bioenergetics and mitochondrial respiration in cultured cells using optical sensors that measure the OCR in cells attached to the culture plate (Ferrick et al., 2008) (**Figure 2.1**). For this experiment, astrocytes were purified as described previously and were counted using the Triton Blue exclusion method. Astrocytes were seeded in specialised XF24-well cell culture plates at a density of 40×10^3 cells per well in 150 µl cDMEM. These plates contain wells for background correction which were left free of cells. Cells were allowed to adhere for 1 h before being provided with an additional 150 µl cDMEM. The cells were left overnight and were treated the following day in a volume of 500 µl with L-AAA (0.05, 0.5, 5 mM) diluted in cDMEM or control (cDMEM alone) for 24 h. On the day prior to OCR analysis, the sensor cartridge of the Seahorse apparatus was hydrated by adding Seahorse XF Calibrant solution (1 ml) to each well of the utility plate and was left overnight in a no-CO₂ incubator at 37°C, 5% CO₂. On the following day, the astrocytes were removed from the incubator and the treatment media was removed. Cells were washed in un-buffered DMEM supplemented with 10 mM Glucose for 1 h at 37 °C in a 21% O₂ incubator before transfer to the XF24 analyser. Experiments consisted of 3 min mixing, 2 min wait, and 3 min measurement cycle. Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitors 0.5 µM oligomycin which inhibits ATP synthase, or in the presence of 0.5 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) - the mitochondrial uncoupler - to assess maximal oxidative capacity. 10 µM rotenone and 5.0 µM antimycin A were injected to give a blank rate. All experiments were performed at 37°C. Basal rates for OCR were calculated by subtracting initial rates from blank rates. Data presented are the mean ± SEM corrected to 1×10^6 cells.

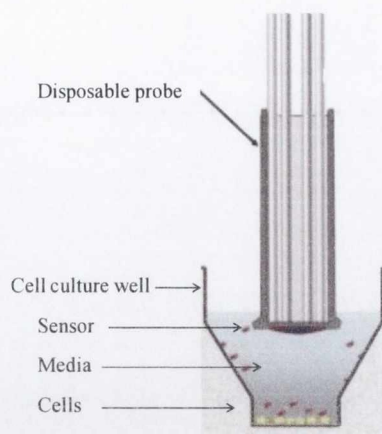


Figure 2.1: Layout of the XF24-well cell culture plates for measurement of oxygen consumption rate using Seahorse apparatus (Biosciences). A cutaway view of a well of the XF24 cell culture plate and sensor is shown above. Image adapted from Seahorse (Biosciences).

2.2.9 Fluorescent immunocytochemistry for glial cell marker staining and Sholl analysis

Fluorescent immunocytochemistry was performed on astrocytes and neurons grown on glass coverslips. All antibodies were diluted in 1 x DPBS (see **Table 2.1** for a list of antibodies used). Cells were washed under sterile conditions twice with 1 x PBS, fixed with ice-cold methanol (300 μ l) for 15 min at -20°C and washed three times with 1 ml 1 x PBS. Following fixation, all subsequent steps were performed on the normal laboratory bench. Non-reactive sites were blocked for 2 h at room temperature in 300 μ l blocking buffer (4% normal goat serum in 1 x PBS). The blocking buffer was then removed and 300 μ l of primary antibody was immediately added. The cells were incubated in primary antibody overnight at 4°C . The next day, cells were washed three times in 1 ml 1 x PBS. Cells were incubated with an appropriate secondary antibody (200 μ l) diluted in 1 x PBS, in a light-protected environment for 2 h at room temperature. Next, cells were washed three times in 1 ml 1 x PBS. The glass coverslips were then removed and mounted onto glass slides with Vectashield fluorescent mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Each coverslip was sealed in nail varnish and stored in the dark at 4°C until visualised with a fluorescence microscope.

2.2.10 Fluorescent immunocytochemistry for the quantification of co-localised synaptophysin and post-synaptic density-95 (PSD-95) expression in synaptic puncta

For these studies, neurons (DIV 20) were treated for 24 h and subsequently fixed in methanol as described above. Non-reactive sites were blocked for 30 min at room temperature in 300 μ l blocking buffer (50% normal goat serum in 1 x PBS) with 0.2% Triton-X-100. The blocking buffer was then removed, cells were rinsed in 1 ml 1 x PBS and 300 μ l of primary antibody cocktail (synaptophysin and post-synaptic density-95 (PSD-95)) was added. The cells were incubated in primary antibody overnight at 4°C . The next day, cells were washed three times in

1ml 1 x PBS and stained with the appropriate secondary antibody (200 µl) diluted in 1 x PBS, in a light-protected environment for 2 h at room temperature. The glass coverslips were then removed and mounted onto glass slides with Vectashield fluorescent mounting media containing 4',6-diamidino-2-phenylindole (DAPI). Each coverslip was sealed in nail varnish and stored in the dark at 4°C until visualised with a fluorescence microscope.

2.2.11 Image analysis for Sholl experiments

The Sholl analysis procedure was adapted from Sholl (1953) and Gutierrez and Davies (2007). For this procedure, β -III tubulin-stained neuronal coverslips were visualised at x200 magnification on a Axio Imager Z1 epifluorescent microscope equipped with a CCD camera and Axiovision software. Coverslips included in Sholl analysis displayed neurons with a healthy network phenotype throughout the entire coverslip considered. Only individual neurons, i.e. neurons that were not in contact with any other neurons, were imaged to isolate neurites and prevent misreading of neuritic length and neurite number. Five to seven individual neurons from each coverslip and eight coverslips per experimental condition were typically imaged and analysed for Sholl analysis. Where possible, groups were blinded. Neuronal morphology was analysed using the following parameters: number of primary neurites, number of neuritic branches, neuritic length and Sholl profile (Sholl 1953). In the classic Sholl analysis, a number of concentric rings with regular radial increments centred in the neuronal soma are traced and the number X_i of neurites intersecting each ring of increasing radii is counted. In this thesis, a semi-automated procedure was used to examine neurite outgrowth and generate a Sholl profile (Gutierrez and Davies, 2007). The same information was derived by reconstruction of the complete pattern of intersections between neurites and concentric rings. The total number of neurites for any segment was calculated as follows: $X_i = X_{i-1} + B_i - T_i$, where X_i is the number of neurites for the “ith” segment, B_i the number of branching events occurring in the “ith” segment and T_i the number of branching terminations occurring in the “ith” segment. This equation was implemented into the programming platform Matlab to reduce analysis time and thereby allow analysis of larger samples of neurons. The inter-ring interval was set at 10 µm and 20 concentric rings were included for analysis. Primary neurites were classified as those directly stemming from the cell body within the first circle, while a branch was counted if a neurite clearly divided in two for at least 5 µm. Neuritic length was defined as the total length of all neurites and was acquired by correlating the manually measured length (µm) of a sample of control-treated neurons against the number of intersections derived from the modified procedure (Fastsholl), and using the resulting linear equation as a standard curve to calculate the total (metric) length in micrometers of neuritic arbors in experimental samples of neurons (Gutierrez and Davies, 2007). To generate the Sholl profile, the number of neuritic branches (intersection points) was plotted against the distance from the cell soma. A stereotypical Sholl profile for pharmacologically untreated rat

cortical neurons is depicted below as an example and is consistent with the Sholl profile typically described in the literature (Gutierrez & Davies 2007) (**Figure 2.2**).

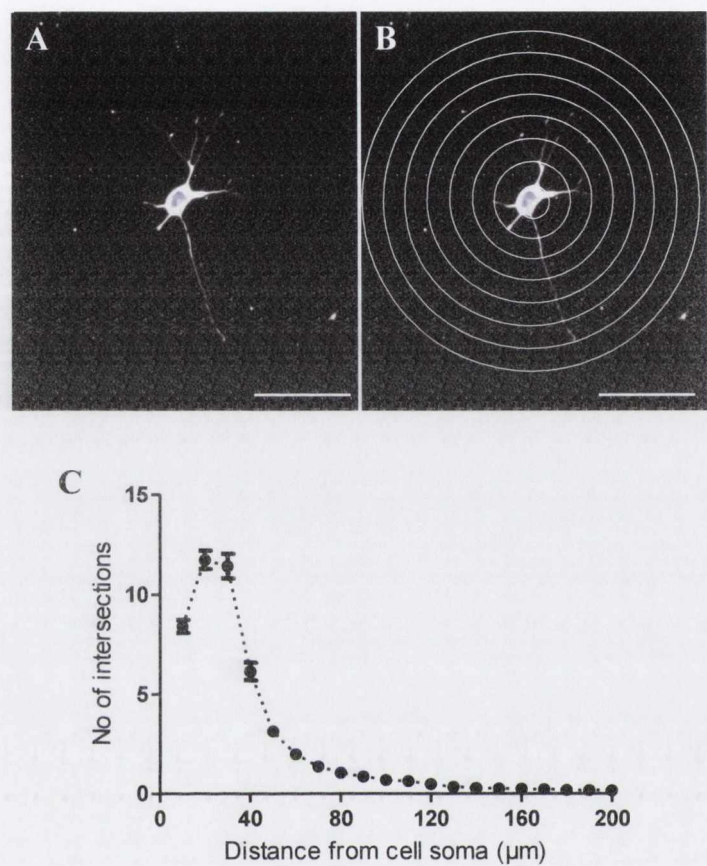


Figure 2.2: Sholl Analysis Procedure. Representative image of a rat cortical neuron stained with β -III tubulin (white) and DAPI (blue) (**A**) with overlaying Sholl analysis concentric rings (**B**). In this example, the neuron is considered to have 5 primary neurites. A typical Sholl profile derived from untreated primary cortical neurons at DIV 4 (**C**). Scale bar =50 μ m, n =24 neurons.

2.2.12 Image analysis for the quantification of co-localised synaptophysin and PSD-95 expression in synaptic puncta

The synaptogenesis procedure was adapted from Ippolito and Eroglu (2010). For this procedure, neurons (DIV 20) were treated for 24 h and were subsequently immunostained with the pre-synaptic marker, synaptophysin and the post-synaptic marker PSD-95 as described above. Synaptophysin and PSD-95 stained neuronal coverslips were visualised at x400 magnification on an Axio Imager Z1 epifluorescent microscope equipped with a CCD camera and Axiovision software. Coverslips included in this analysis displayed neurons with a healthy network phenotype throughout the entire coverslip. Three to five individual images were captured from each coverslip and six coverslips per experimental condition were typically collected and analysed for analysis. Where possible, groups were blinded. In this assay, synapses were defined

as points of co-localisation between the signals generated by pre-synaptic synaptophysin and post-synaptic PSD-95. The number of co-localisations were quantified using a plug-in Puncta Analyser for use in ImageJ analysis software platform. For image analysis, individual images were opened in ImageJ and a region of interest (ROI) was selected which was typically one-cell diameter radially around the soma of interest. The Puncta Analyser was then used to adjust the thresholds of the red channel (synaptophysin expression) and green channel (PSD-95 expression) so that the intensity corresponded to discrete individual puncta. Minimum puncta size was set at a value of 4 for each experiment. The threshold value was standardised for each based on a value established for a control-treated neuron in order to maintain a consistent approach for comparison between groups (**Figure 2.3**).

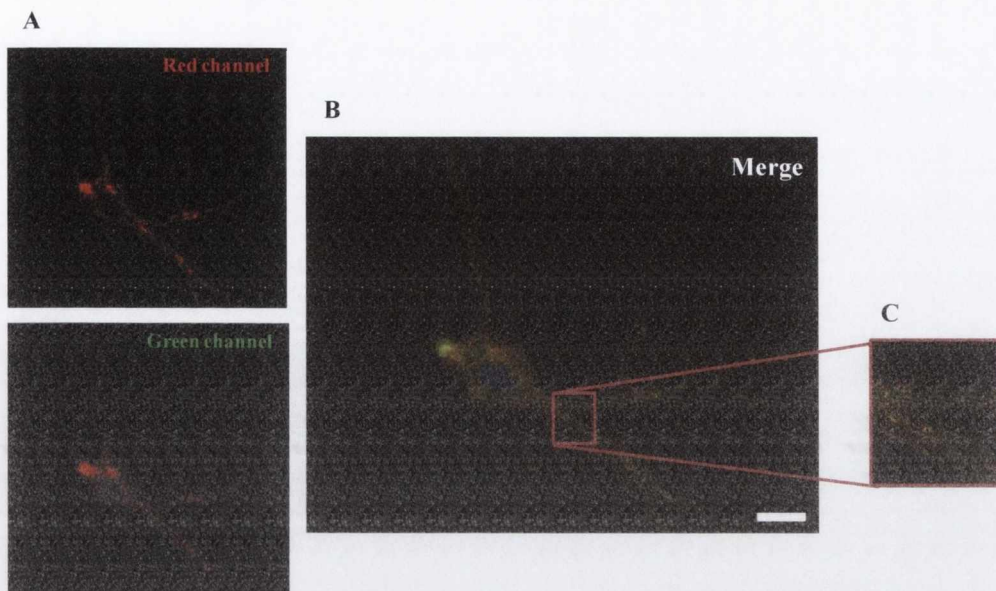


Figure 2.3: Quantification of the co-localised expression of synaptophysin and PSD-95 in synaptic puncta. Primary cortical neurons were treated at DIV 20 for 24 h before fixation. **(A)** Neurons were stained with the pre-synaptic marker, synaptophysin (red channel image) and the post-synaptic marker, PSD-95 (green channel image). **(B)** Merged image showing synaptophysin, PSD-95 and the DAPI nuclear stain (blue). Quantification of synapse number was conducted using ImageJ software where synapses are defined as points of co-localisation between synaptophysin and PSD-95 formation. Scale Bar = 20 μm . **(C)** Individual synaptic co-localised puncta are displayed as black dots in the inset image.

2.2.13 Statistical analysis

Data are expressed as group mean with standard errors and were analysed using unpaired Student's *t* tests or analyses of variance (ANOVA). If a statistically significant change was found following ANOVA, *post hoc* comparisons were performed using Student-Newman-Keuls or Bonferroni tests, where appropriate. Data were deemed significant when $P<0.05$.

Chapter 3: Small-molecule inhibitors at the PSD-95/nNOS interface protect against glutamate-induced neuronal atrophy in primary cortical neurons.

3.1 Introduction

Glutamate is the main excitatory neurotransmitter in the CNS and is important for several aspects of neurite outgrowth, synaptogenesis and neuronal survival in both the developing and adult nervous system (Esquenazi et al., 2002, Mattson, 2008, Selvakumar et al., 2013). During development, glutamate is released by immature, developing neurons prior to synapse formation. In these early stages of development, its effects on neurite outgrowth are thought to be mediated through altering the motility of filopodia in the growth cones of developing neurons through NMDA-R and AMPA-R activation (Kuo et al., 2010). In addition, Ca^{2+} influx mediated by glutamate receptor activation leads to various cellular responses which can result in changes in neurite morphology and outgrowth. For example, glutamate mediated Ca^{2+} influx has been suggested to aid neurite elongation by causing rapid local polymerisation of actin to form filopodial extensions of growth cones, while inhibiting tubulin polymerisation (Mattson, 2008).

It is well established that excessive or sustained glutamate receptor activation is detrimental to neurons and can lead to excitotoxic damage and neuronal atrophy and degeneration (Greenwood and Connolly, 2007, Willard and Koochekpour, 2013). Under normal physiological conditions, active transport systems keep glutamate levels in the extracellular environment within the 1-10 micromolar range. However, under pathological conditions such as epilepsy and ischemia, extracellular concentrations of glutamate can drastically increase to near millimolar levels and contribute to the neurodegeneration observed in these conditions (Esquenazi et al., 2002). The earliest signs of glutamate excitotoxicity involve alterations in dendrite morphology, and these alterations often precede excitotoxic cell death (Monnerie et al., 2003). Additional early signs of glutamate-induced neuronal damage include focal swelling, dendritic beading and spine loss – phenomena which may correspond to reductions in synaptic connections and hence disruptions in neuronal function (Greenwood and Connolly, 2007). Therefore, targeting the early glutamate-induced changes in neurite outgrowth may represent a promising strategy to prevent subsequent cell death and promote neuronal remodelling and functional recovery.

The NMDA-R is functionally coupled to nNOS via the PSD-95 (Christopherson et al., 1999), thereby allowing NMDA-R-mediated Ca^{2+} to activate nNOS and lead to the production of NO. PSD-95 is a synaptic protein which is important in excitatory synapse formation and maturation (El-Husseini et al., 2000) as well as playing a role in the regulation of dendrite outgrowth and branching, independent of its synaptic functions (Charych et al., 2006). NO is also known to play important roles in neurite development (Hess et al., 1993, Hindley et al., 1997) and in the regulation of synaptic plasticity (Garthwaite and Boulton, 1995, Contestabile, 2000). Aside from

these physiological functions, excess production of NO is associated with the production of reactive nitrogen species (RNS) leading to cellular damage and neurotoxicity (Calabrese et al., 2007). In terms of neurite outgrowth, increasing NO levels using NO donors have been reported to facilitate neurotrophin-induced outgrowth (Hindley et al., 1997) but have also been shown to induce axonal retraction by causing growth cone collapse and microtubule reconfiguration (Ernst et al., 2000, He et al., 2002).

In this investigation we were particularly interested in investigating the effects of two small molecular weight inhibitors of the PSD-95/nNOS interaction on NMDA-induced changes in neurite outgrowth and synapse morphology as measured by the co-localisation of pre-synaptic synaptophysin and post-synaptic PSD-95 in synaptic puncta. These inhibitors - IC87201 and ZL006 - have demonstrated efficacy in animal models of neurological disorders associated with excessive glutamatergic neurotransmission. For instance, IC87201 has been shown to inhibit acute thermal hyperalgesia and chronic allodynia in rodent models of pain (Florio et al., 2009), while intravenous ZL006 reduced infarct size and improved neurological scores in a mouse MCAO model of stroke (Zhou et al., 2010). More recently, ZL006 has been shown to promote neuronal differentiation of neural stem cells (NSCs) and enhance neurite growth of newborn neurons and dendritic spine formation of mature neurons in the ischemic brain of rats after MCAO (Luo et al., 2014). Prior research from our laboratory has also demonstrated that IC87201 and ZL006 display antidepressant-like properties in mice in keeping with putative novel neurotrophic properties of these compounds (Doucet et al., 2013).

In the current investigation, we assessed the effects of glutamatergic stimulation and NO production on neurite outgrowth and the co-localised expression of synaptic proteins in cultured neurons. The NMDA-R antagonists ketamine and MK-801 were investigated for their potential to protect against NMDA/glycine-induced neurite shrinkage and potential synapse loss. Targets downstream of the NMDA-R, such as nNOS and soluble guanylate cyclase (sGC), may also impact upon glutamate-mediated atrophy. In this regard, the nNOS inhibitor 1-[2-(trifluoromethyl)phenyl] imidazole (TRIM) was tested for effects on neurite outgrowth and synapse number following exposure of neurons to NMDA and the NO precursor, L-arginine. Moreover, the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was assessed for its impact on L-arginine-mediated reductions in neurite outgrowth. Finally, we hypothesised that uncoupling nNOS from the NMDA-R with the PSD-95/nNOS inhibitors IC87201 and ZL006 would attenuate the NMDA/glycine-induced loss of neuronal complexity. To test the specificity of these inhibitors in blocking the PSD-95/nNOS interaction, we hypothesised that they would be unable to attenuate downstream NO-mediated reductions in neurite outgrowth by assessing the ability of IC87201 to prevent reductions in outgrowth induced by L-arginine.

3.2 Study designs

3.2.1 Concentration-related effects of glutamate, glutamate/glycine and NMDA/glycine on neurite outgrowth

Primary cortical neurons (DIV 3) were treated with glutamate (100 and 500 μM) or with a combination of glutamate (100 and 500 μM) and glycine (10 μM) for 24 h before fixation and immunocytochemistry for Sholl analysis. Glycine was added to glutamate on the basis that it acts as an essential co-agonist at NMDA-Rs (Dingledine et al., 1999). Concentrations of glutamate were based on the fact that glutamate (100 μM) saturates all types of glutamatergic ionotropic receptors in neuronal cultures (Metzger et al., 1998). For comparison purposes with glutamate, primary cortical neurons (DIV 3) were treated with a combination of NMDA (100, 250 and 500 μM) and glycine (10 μM) for 24 h before fixation and immunocytochemistry for Sholl analysis. NMDA effects were examined on the basis that NMDA is a more selective agonist for the NMDA-R subtype than glutamate. Concentrations of glutamate and NMDA were based on their deleterious effects on neurite outgrowth reported from previous studies (Esquenazi et al., 2002, Monnerie et al., 2003).

3.2.2 Concentration-related effects of SNP and L-arginine on neurite outgrowth

Primary cortical neurons (DIV 3) were treated with SNP (30, 100, 300 μM) or L-arginine (30, 100, 300 μM) for 24 h before fixation and immunocytochemistry for Sholl analysis. Concentrations of SNP were based on previously published reports indicating their effects on neurons *in vitro* over a similar range of concentrations (Dawson et al., 1991). Concentrations of L-arginine were evaluated at the same concentrations for comparative purposes.

3.2.3 Effects of NBQX on glutamate-induced neurite outgrowth

Primary cortical neurons (DIV 3) were pre-treated with glutamate (100 μM) for 1 h followed by treatment with a combination of glutamate (100 μM) and NBQX (10 μM) for 23 h before fixation and immunocytochemistry for Sholl analysis. The effects of the AMPA-R antagonist NBQX were examined to further dissect the respective roles of AMPA-Rs and NMDA-Rs in glutamate-induced neurite outgrowth. The concentration of NBQX was based on previous reports using the related AMPA-R/kainate receptor antagonist CNQX (10 μM) to restrict the actions of applied glutamate to NMDA-Rs and prevent the activation of AMPA-Rs (Aarts et al., 2002, Maeng and Zarate, 2007) and on the fact that NBQX (3 μM) was able to reverse glutamate-induced reduction in neurite outgrowth in motoneurons following culture and treatment for 5 days (Metzger et al., 1998).

3.2.4 Effects of MK-801 and ketamine on NMDA/glycine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and MK-801 (10 nM) or ketamine (10 nM) for 23 h before fixation and immunocytochemistry for Sholl analysis. Ketamine concentrations were selected on the basis that ketamine potentiated NGF-induced neurite outgrowth in PC12 cells using similar concentrations (Robson et al., 2012). The effects of MK-801 were evaluated at the same concentration for comparative purposes.

3.2.5 Effects of TRIM on NMDA/glycine- and L-arginine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) or L-arginine (300 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) or L-arginine (300 μ M) and TRIM (100 nM) for 23 h before fixation and immunocytochemistry for Sholl analysis.

3.2.6 Effects of ODQ on L-arginine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were treated with L-arginine (300 μ M) for 1 h followed by treatment with a combination of the soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μ M) and L-arginine (300 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis. The concentration of ODQ was chosen based on previous reports which used this concentration on rodent neurons in culture (Yun et al., 1998, Huang et al., 2003).

3.2.7 Effects of IC87201 on NMDA/glycine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and IC87201 (10 nM or 100 nM) for 23 h before fixation and immunocytochemistry for Sholl analysis. The selection of concentrations was determined from previous published reports indicating that IC87201 dose-dependently reduced NMDA-induced cGMP production in primary hippocampal neurons (DIV 14-21) with an IC₅₀ of 2.7 μ M and that IC87201 (1 mg/kg) was effective in treating NMDA-induced thermal hyperalgesia in mice, with a corresponding peak plasma level of 55 ng/mL (or 0.2 μ M) (Florio et al., 2009). Antidepressant-like effects were obtained over a similar range of doses (1-2 mg/kg) in mice (Doucet et al., 2013).

3.2.8 Effects of ZL006 on NMDA/glycine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and ZL006 (10 nM or 100 nM) for 23 h before fixation and immunocytochemistry for Sholl analysis. The concentrations were selected on the basis that ZL006 was previously shown to inhibit NMDA-induced PSD-95/nNOS binding in cortical neurons (DIV 7-9) with an IC_{50} of 82 nM following treatment with glutamate (50 μ M) and glycine (10 μ M) for 30 min (Zhou et al., 2010).

3.2.9 Effects of IC87201 on L-arginine-induced reduction in neurite outgrowth

Primary cortical neurons (DIV 3) were treated with L-arginine (300 μ M) for 1 h followed by treatment with a combination of IC87201 (100 nM) and L-arginine (300 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis.

3.2.10 Concentration-related effects of NMDA on neurite outgrowth of mature (DIV 20) neuronal cultures

In the following studies, the effects of NMDA and L-arginine on the expression of co-localised proteins in synaptic puncta were investigated. Synapse detection can be achieved *in vitro* through immunostaining using a pre-synaptic marker, such as synaptophysin, with a post-synaptic marker, such as PSD-95, and the quantification of co-localisation points between these two markers are taken as a measure of synapse number (Ippolito and Eroglu, 2010, Roqué et al., 2014). As the majority of synapse formation occurs in mammalian neurons in culture between week 2 and 3 (Nimchinsky et al. 2002), the neurons for synaptic protein studies were cultured until DIV 20 before treatment. More mature neurons have higher sensitivity to the effects of glutamate than immature neurons (Monnerie et al., 2003), and it was important to firstly establish a concentration of NMDA for these studies. Since a reduction in neurite length would likely lead to a concomitant reduction in the number of synapses present on a given neurite, we controlled for this by determining a concentration of NMDA that would not lead to overt reductions in neurite outgrowth but may affect synaptic protein co-localisation.

3.2.11 Effects of MK-801, ketamine and TRIM on NMDA/glycine-induced reductions in the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

Primary cortical neurons (DIV 20) were pre-treated with NMDA (10 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (10 μ M)/glycine (10 μ M) and MK-801 (10 nM), ketamine (10 nM) or TRIM (100 nM) for 23 h before fixation and immunocytochemistry for analysis of the number of co-localised synaptophysin and PSD-95 puncta. Primary cortical

neurons (DIV 20) were also pre-treated with L-arginine (10 μ M) for 1 h followed by treatment with a combination of L-arginine (10 μ M) and TRIM (100 nM) for 23 h before fixation and immunocytochemistry for analysis of the number of co-localised synaptophysin and PSD-95 synaptic puncta.

3.2.12 Effects of ZL006 and IC87201 on NMDA/glycine-induced reductions in the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

Primary cortical neurons (DIV 20) were pre-treated with NMDA (10 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (10 μ M)/glycine (10 μ M) and ZL006 (100 nM), or IC87201 (100 nM) for 23 h before fixation and immunocytochemistry for analysis of the number of co-localised synaptophysin and PSD-95 synaptic puncta.

3.3 Results

3.3.1 Distribution of PSD-95 and nNOS in primary cortical neurons at DIV 4

Prior to investigating the effects of PSD-95 and nNOS modulation on neurite outgrowth responses, immunocytochemistry was performed to examine the expression of PSD-95 and nNOS in cultured primary cortical neurons at DIV4. PSD-95 was localised in neurites and soma, particularly in the perinuclear region with punctuate expression. nNOS co-localised with PSD-95 with strong expression in the neurites and soma. This distribution suggests that cortical neurons may receive glutamate signals from the soma, dendrites or even the axon to regulate neurite outgrowth and branching (Kuo et al., 2010). Taken together, these results demonstrate that PSD-95 and nNOS are expressed and co-localised in immature cortical neurons, and are organised to play a role in regulating glutamate-mediated neurite outgrowth (**Figure 3.1**).

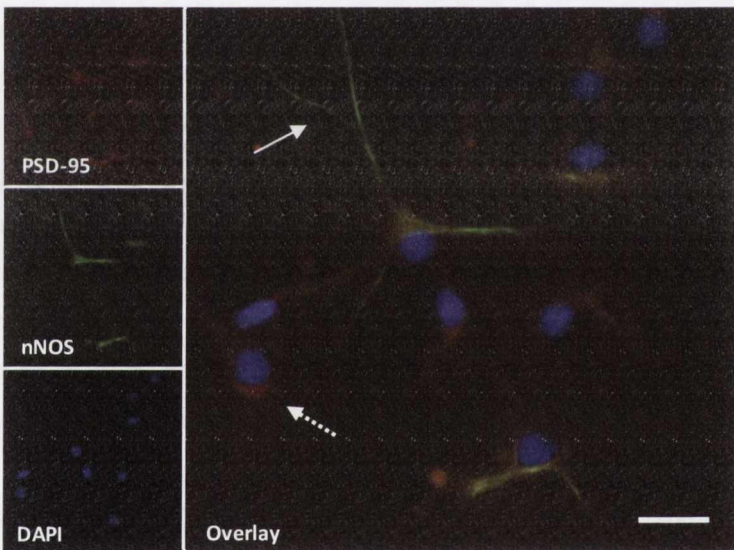


Figure 3.1: Immature cortical neurons express PSD-95 and nNOS. Immunocytochemistry was performed on cortical neuronal cultures to verify that immature neurons (DIV 4) expressed PSD-95 and nNOS. Neurons were identified using PSD-95 (red), nNOS (green) and DAPI (blue). PSD-95 was localised in neurites and in the perinuclear region of the cell body (white dashed arrow), while nNOS was strongly distributed in neurites and cell body (white arrow). Scale bar, 20 μ m.

3.3.2 Concentration-related effects of glutamate, glutamate/glycine and NMDA/glycine on neuronal complexity

Prior research has demonstrated that glutamate can both increase and decrease dendrite outgrowth in hippocampal pyramidal neurons *in vitro* (Mattson et al., 1988, Wilson and Keith, 1998). Here, we examined the concentration-related effects of glutamate and NMDA on cortical neurite outgrowth at DIV 3-4.

3.3.2.1 Concentration-related effects of glutamate

One-way ANOVA demonstrated a significant effect of glutamate on the number of primary neurites ($F_{2,65}=3.93$, $P=0.025$), neuritic length ($F_{2,65}=8.10$, $P<0.001$) and number of neuritic branches ($F_{2,65}=7.22$, $P=0.0015$). *Post hoc* analysis revealed that glutamate (100 μM) increased neuritic length ($P<0.01$) and number of neuritic branches ($P<0.05$) when compared to control-treated neurons. Furthermore, two-way repeated measures ANOVA demonstrated an effect of glutamate on the number of neuritic branches at specific distances from the neuronal cell soma ($F_{2,1235}=8.53$, $P<0.001$). ANOVA also demonstrated an effect of distance ($F_{19,1235}=733.3$, $P<0.001$) and a glutamate x distance interaction ($F_{38,1235}=3.65$, $P<0.001$). *Post hoc* analysis revealed that neurons treated with glutamate (100 μM) had more branches than control-treated neurons at 10-50 μm ($P<0.01$). In contrast, higher concentrations of glutamate (500 μM) decreased the number of neuritic branches at 20 μm ($P<0.01$) when compared to controls (**Figure 3.2A**).

3.3.2.2 Concentration-related effects of glutamate/glycine

One-way ANOVA demonstrated an effect of treatment on neuritic length ($F_{3,89}=2.94$, $P=0.038$) but failed to demonstrate any effect on the number of primary neurites and neuritic branches. *Post hoc* analysis revealed that glutamate (500 μM)/glycine (10 μM) decreased neuritic length ($P<0.05$) when compared to control-treated neurons. Furthermore, two-way repeated measures ANOVA demonstrated an effect of treatment on the number of neuritic branches at specific distances from the neuronal cell soma ($F_{3,1691}=2.75$, $P=0.047$). ANOVA also demonstrated an effect of distance ($F_{19,1691}=650.2$, $P<0.001$) but failed to demonstrate a treatment x distance interaction. *Post hoc* analysis revealed that neurons treated with glutamate (100 μM)/glycine (10 μM) and glutamate (500 μM)/glycine (10 μM) had fewer branches than control-treated neurons at 20 μm ($P<0.05$), 30 and 40 μm ($P<0.01$) (**Figure 3.2B, D**).

3.3.2.3 Concentration-related effects of NMDA/glycine

One-way ANOVA demonstrated an effect of NMDA/glycine treatment on the number of primary neurites ($F_{4,112}=3.34$, $P=0.013$) and neuritic length ($F_{4,112}=6.81$, $P<0.001$) *Post hoc* analysis revealed that NMDA (250 μM)/glycine (10 μM) and NMDA (500 μM)/glycine (10 μM)

decreased neuritic length ($P<0.01$) when compared to control-treated neurons. One-way ANOVA also demonstrated an effect of treatment on the number of neuritic branches ($F_{4,112}=5.43$, $P<0.001$). *Post hoc* analysis revealed that the three concentrations of NMDA tested (100, 250 and 500 μM) combined with glycine (10 μM) decreased the number of neuritic branches ($P<0.05$, $P<0.01$ and $P<0.01$, respectively) when compared to control-treated neurons. Furthermore, two-way repeated measures ANOVA demonstrated an effect of treatment on the number of neuritic branches at specific distances from the neuronal cell soma ($F_{4,2128}=7.00$, $P<0.001$). ANOVA also demonstrated an effect of distance ($F_{19,2128}=1006$, $P<0.001$) and a treatment x distance interaction ($F_{76,2128}=4.06$, $P<0.001$). *Post hoc* analysis revealed that neurons treated with NMDA (100 μM)/glycine (10 μM) had fewer branches than control-treated neurons at 20 μm ($P<0.05$), 30 and 40 μm ($P<0.01$) and 50 μm ($P<0.05$). When the concentration of NMDA was increased to 250 or 500 μM , neurons had significantly fewer branches than control-treated neurons at 10 μm ($P<0.05$), 20, 30, 40 and 50 μm ($P<0.01$ at both concentrations) (**Figure 3. 2C, D**).

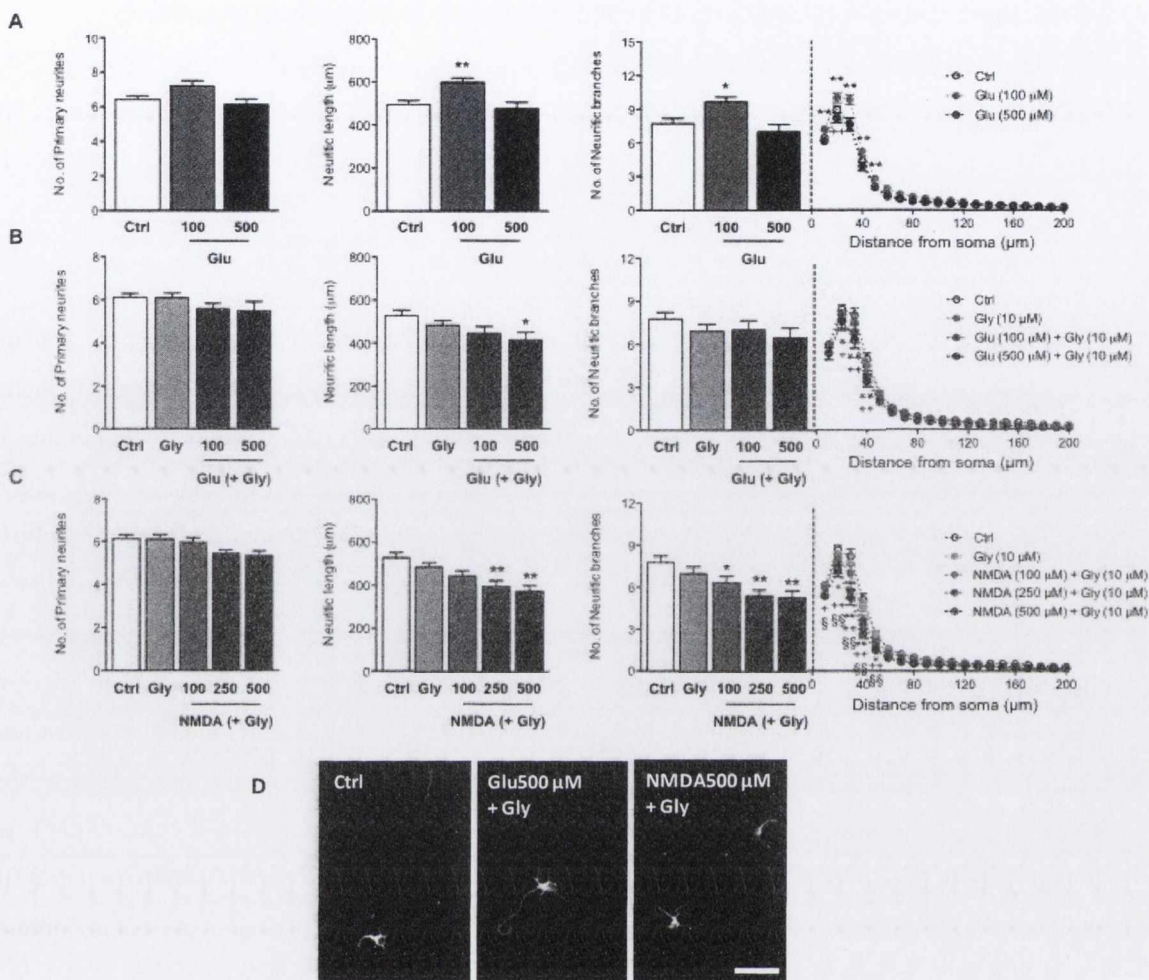


Figure 3.2: Concentration-related effects of glutamate, glutamate/glycine and NMDA/glycine on neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with (A) glutamate (100 and 500 μ M), (B) a combination of glutamate (100 and 500 μ M) and glycine (10 μ M) or (C) a combination of NMDA (100, 250 and 500 μ M) and glycine (10 μ M). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. (D) Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μ m. Data are expressed as mean $\pm$ SEM, $n=21-24$ coverslips per group representative of 3 independent experiments. ** $P<0.01$ and * $P<0.05$ vs. ctrl (Bonferonni's or Student-Newman-Keuls); Sholl profile, ** $P<0.01$ and * $P<0.05$ 100 μ M vs. ctrl, §§ $P<0.01$ and § $P<0.05$ 250 μ M vs. ctrl, ++ $P<0.01$ and + $P<0.05$ 500 μ M vs. ctrl (Student-Newman-Keuls).

3.3.3 Concentration-related effects of SNP and L-arginine on neuronal complexity

Since excess glutamate and NMDA caused reductions in neurite outgrowth, and nitric oxide (NO) production acts down-stream of NMDA-R activation, the effects of increased production of NO were investigated, using the NO donor sodium nitroprusside (SNP) and the NO precursor L-arginine (L-arg).

3.3.3.1 Concentration-related effects of SNP

One-way ANOVA demonstrated a significant effect of SNP on the number of primary neurites ($F_{3,92}=5.54$, $P=0.0015$), neuritic length ($F_{3,29}=27.82$, $P<0.001$) and number of neuritic branches ($F_{3,92}=16.40$, $P<0.001$). *Post hoc* analysis revealed that SNP (300 μ M) decreased the number of primary neurites ($P<0.05$). SNP (30 μ M) decreased neuritic length ($P<0.05$), with higher concentrations (100 μ M and 300 μ M) resulting in further reductions to neuritic length ($P<0.001$). SNP (100 μ M and 300 μ M) also decreased the number of neuritic branches ($P<0.001$) compared to control-treated neurons. Furthermore, two-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated an effect of distance ($F_{19,1748}=1219$, $P<0.001$), treatment ($F_{3,1748}=23.34$, $P<0.001$) and a distance x treatment interaction ($F_{57,1748}=1.47$, $P<0.01$) on the number of neuritic branches at specific distances from the neuronal cell soma. *Post hoc* analysis revealed that neurons treated with SNP (300 μ M) had fewer branches than control-treated neurons at 10-30 μ m ($P<0.001$) and 40-200 μ m ($P<0.01$) while neurons treated with SNP (100 μ M) had significantly fewer branches than control-treated neurons at 150-180 μ m ($P<0.01$) (**Figure 3.3A, C**).

3.3.3.2 Concentration-related effects of L-arginine

One-way ANOVA demonstrated an effect of L-arginine on the number of primary neurites ($F_{3,79}=4.52$, $P=0.005$), the neuritic length ($F_{3,79}=3408$, $P=0.022$) and the number of neuritic branches ($F_{3,79}=6.30$, $P<0.001$). *Post hoc* analysis revealed that L-arginine (300 μ M) decreased the number of primary neurites ($P<0.01$), neuritic length ($P<0.05$), and number of neuritic branches ($P<0.01$) compared to control-treated neurons. Furthermore, two-way repeated measures ANOVA demonstrated an effect of L-arginine on the number of neuritic branches at specific distances from the neuronal cell soma ($F_{3,1501}=3.23$, $P=0.027$). ANOVA also demonstrated an effect of distance ($F_{19,1501}=733$, $P<0.0001$) and a L-arginine x distance interaction ($F_{57,1501}=2.031$, $P<0.0001$). *Post hoc* analysis revealed that neurons treated with L-arginine (300 μ M) had fewer branches than control-treated neurons at 10-30 μ m ($P<0.001$) (**Figure 3.3B, C**).

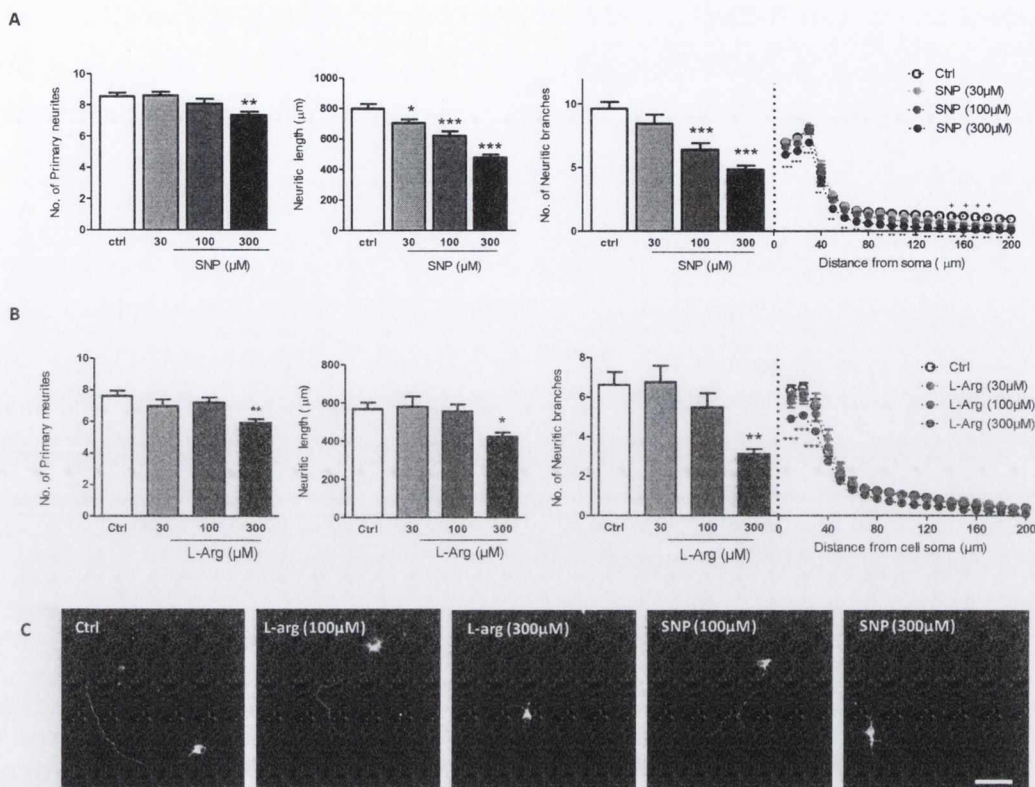


Figure 3.3: Concentration-related effects of SNP and L-arginine on neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with (A) SNP (30, 100, 300 μ M) or (B) L-arginine (30, 100, 300 μ M). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. (C) Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μ m. Data are expressed as mean $\pm$ SEM, n=20-24 coverslips per group representative of 3 independent experiments. ***P<0.001, **P<0.01, *P<0.05 vs. ctrl (Student-Newman-Keuls); Sholl profile, ***P<0.001, **P<0.01, *P<0.05 SNP or L-arginine (300 μ M) vs. ctrl, +++P<0.001, ++P<0.01, +P<0.05 SNP or L-arginine (100 μ M) vs. ctrl (Bonferroni).

3.3.4 Effects of NBQX on glutamate-induced neurite outgrowth

Since glutamate (100 μ M) promoted neurite outgrowth and the glutamate (100 μ M)/glycine (10 μ M) combination attenuated this increase, we hypothesised that glutamate may promote neurite outgrowth via a mechanism of action involving AMPA-Rs, while NMDA-Rs may exert opposite effects on neurite outgrowth. To further examine this hypothesis, rat cortical neurons were treated with glutamate (100 μ M) and the AMPA-R antagonist NBQX (10 μ M).

Two-way ANOVA demonstrated an effect of glutamate (Glu) pre-treatment on number of neuritic branches ($F_{1,86}=8.14$, $P=0.0054$) and neuritic length ($F_{1,86}=4.15$, $P=0.045$). ANOVA also

demonstrated an effect of NBQX treatment on the number of neuritic branches ($F_{1,86}=5.00$, $P=0.028$) but there was no glutamate x NBQX interaction. *Post hoc* analysis revealed that glutamate treatment increased the number of neuritic branches ($P<0.05$), which was blocked by NBQX treatment ($P<0.05$). In addition, NBQX treatment decreased neuritic length ($P<0.05$) in glutamate treated primary cortical neurons. Furthermore, three-way repeated measures ANOVA demonstrated an effect of glutamate pre-treatment ($F_{1,86}=4.73$, $P=0.032$) on the number of neuritic branches at specific distances from the neuronal cell soma. ANOVA also demonstrated an effect of distance ($F_{19,1634}=1090$, $P<0.001$) and a NBQX treatment x distance interaction ($F_{19,1634}=3.88$, $P<0.001$). *Post hoc* analysis revealed that neurons treated with glutamate (100 μ M) had more branches than control-treated neurons at 20 μ m ($P<0.01$) and 30 μ m ($P<0.05$), while neurons treated with glutamate (100 μ M)/NBQX (10 μ M) had fewer branches than glutamate-treated neurons at 20 and 30 μ m ($P<0.01$) (**Figure 3.4A**).

3.3.5 Effects of MK-801 and ketamine on NMDA-induced reduction in neurite outgrowth

Since the combination of NMDA (250 μ M) and glycine (10 μ M) decreased neurite outgrowth, the data supports the hypothesis that over-activation of the NMDA-R subtype has deleterious effects on neurite outgrowth. To further investigate this hypothesis, the potential of the NMDA-R antagonists MK-801 (10 nM) and ketamine (10 nM) to reverse the effects of NMDA/glycine-induced reduction in neurite outgrowth were assessed.

3.3.5.1 Effects of MK-801 on NMDA-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,76}=11.06$, $P<0.001$), MK-801 ($F_{1,76}=17.59$, $P<0.001$) and an NMDA/Gly x MK-801 interaction ($F_{1,76}=9.51$, $P=0.003$) on the neuritic length and an effect of MK-801 ($F_{1,76}=9.4$, $P<0.003$) and NMDA/Gly x MK-801 interaction ($F_{1,76}=12.41$, $P<0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA-treated neurons had decreased neuritic length and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons. Treatment with MK-801 was able to reverse these deficits, as evidenced by increased neuritic length and branch number ($P<0.01$) of neurons treated with NMDA/Gly and MK-801 when compared to NMDA/Gly-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of distance ($F_{19,1444}=14.36$, $P<0.001$), NMDA/Gly x distance interaction ($F_{19,1444}=18.05$, $P<0.001$), MK-801 x distance interaction ($F_{19,1444}=12.4$, $P<0.001$) and NMDA/Gly x MK-801 x distance interaction ($F_{19,1444}=8.51$, $P<0.001$). *Post hoc* analysis revealed that MK-801 increased the number of branches at 10, 20, 50 and 60 μ m ($P<0.01$) when compared to control-treated neurons. In contrast, NMDA/Gly

decreased the number of branches at 10, 20, 50, 70 and 80 μm ($P<0.01$) and 100 μm ($P<0.05$) when compared to control-treated neurons. MK-801 was able to reverse this decrease at 10-70 μm ($P<0.01$) (**Figure 3.4B**).

3.3.5.2 Effects of ketamine on NMDA-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of ketamine ($F_{1,134}=23.71$, $P<0.001$) and an NMDA/Gly x ketamine interaction ($F_{1,134}=5.60$, $P=0.019$) on the number of primary neurites and an effect of NMDA/Gly ($F_{1,134}=21.36$, $P<0.001$) and NMDA/Gly x ketamine interaction ($F_{1,134}=5.41$, $P=0.02$) on the number of neuritic branches. Two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,134}=6.07$, $P=0.015$), ketamine ($F_{1,134}=26.00$, $P<0.001$) and NMDA/Gly x ketamine interaction ($F_{1,134}=19.16$, $P<0.001$) on neuritic length. *Post hoc* analysis revealed that NMDA-treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with ketamine was able to reverse these deficits, as evidenced by increased number of primary neurites, neuritic length and branches ($P<0.01$) of neurons treated with NMDA/Gly and ketamine when compared to NMDA/Gly-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,134}=11.17$, $P=0.0011$), ketamine ($F_{1,134}=50.02$, $P<0.001$), NMDA/Gly x ketamine interaction ($F_{1,134}=26.63$, $P<0.001$), distance ($F_{19,2546}=2032$, $P<0.001$), NMDA/Gly x distance interaction ($F_{19,2546}=1.66$, $P=0.037$), ketamine x distance interaction ($F_{19,2546}=16.88$, $P<0.001$) and NMDA/Gly x ketamine x distance interaction ($F_{19,2546}=6.24$, $P<0.001$). *Post hoc* analysis revealed that ketamine increased the number of branches at 10-30 μm ($P<0.01$) when compared to control-treated neurons. In contrast, NMDA/Gly decreased the number of branches at 10-50 μm ($P<0.01$) and 60 μm ($P<0.05$) when compared to control-treated neurons. Ketamine was able to reverse this decrease at 10-50 μm ($P<0.01$) but not at 60 μm (**Figure 3.4C**).

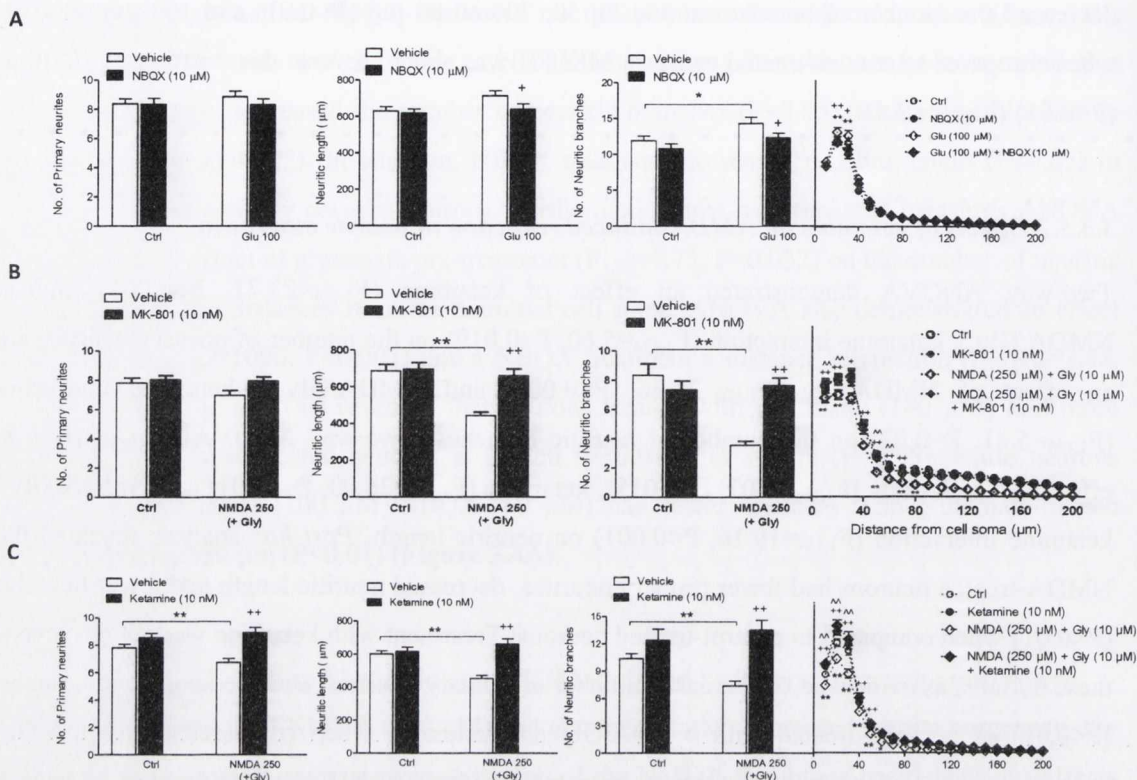


Figure 3.4: Effects of NBQX on glutamate-induced neurite outgrowth and effects of MK-801 and ketamine on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with (A) glutamate (100 μ M) and NBQX (10 μ M), (B) a combination of NMDA (250 μ M)/glycine (10 μ M) and MK-801 (10 nM) or (C) a combination of NMDA (250 μ M)/glycine (10 μ M) and ketamine (10 nM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. Data are expressed as mean $\pm$ SEM, n=20-39 coverslips per group representative of 3-5 independent experiments. **P<0.01 and *P<0.05 vs. ctrl, ++P<0.01 and +P<0.05 vs. respective vehicle (Student-Newman-Keuls); Sholl profile, **P<0.01, *P<0.05 Glu100 vs. ctrl and NMDA250/Gly vs. ctrl, ++P<0.01 Glu100/NBQX vs. Glu100 and NMDA250/Gly/MK-801 vs. NMDA250/Gly and NMDA250/Gly/Ket vs. NMDA250/Gly. ^^P<0.01 MK-801/Ket10 vs. ctrl (Student-Newman-Keuls).

3.3.6 Effects of TRIM on NMDA/glycine and L-arginine-induced reduction in neurite outgrowth

Considering the ability of the NMDA-R antagonists ketamine and MK-801 (10 nM) to protect against NMDA/glycine-induced reduction in neurite outgrowth, the nNOS inhibitor TRIM (100 nM) was also assessed for its potential to counteract both NMDA and L-arginine-induced reductions in neurite outgrowth.

3.3.6.1 Effect of TRIM on NMDA-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of TRIM ($F_{1,58}=15.72$, $P<0.001$) and an NMDA/Gly x TRIM interaction ($F_{1,58}=11.69$, $P=0.0012$) on neuritic length. *Post hoc* analysis revealed that NMDA-treated neurons had reduced neuritic length ($P<0.01$) when compared to control-treated neurons. Treatment with TRIM was able to reverse these deficits in neuritic length, as evidenced by increased neuritic length ($P<0.01$) of neurons treated with NMDA/Gly and TRIM when compared to NMDA/Gly-treated neurons. With regard to number of neuritic branches, two-way ANOVA demonstrated an effect of TRIM ($F_{1,57}=5.16$, $P=0.003$) and a TRIM x NMDA/Gly interaction ($F_{1,57}=12.52$, $P<0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that neurons treated with NMDA/Gly had fewer neuritic branches compared to control and that neurons treated with a combination of TRIM and NMDA/Gly had more branches compared to NMDA/Gly treated neurons alone ($P<0.01$). Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,52}=14.84$, $P<0.001$), NMDA/Gly x TRIM (100 nM) ($F_{1,52}=13.35$, $P<0.001$), distance ($F_{19,988}=752.29$, $P<0.001$), NMDA/Gly x distance ($F_{19,988}=2.45$, $P=0.0042$), TRIM x distance ($F_{19,988}=2.449$, $P=0.0005$). *Post hoc* analysis revealed that NMDA/Gly decreased the number of branches at 10-30 μm ($P<0.01$) when compared to control-treated neurons. Treatment with TRIM (100 nM) reversed this decrease at 10-30 μm ($P<0.01$) (**Figure 3.5A**).

3.3.6.2 Effect of TRIM on L-arginine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of L-arginine ($F_{1,48}=20.03$, $P<0.001$) and TRIM ($F_{1,48}=4.33$, $P=0.04$) on the number of primary neurites and a significant effect of L-arginine ($F_{1,48}=17.25$, $P<0.001$) and TRIM ($F_{1,48}=7.30$, $P=0.009$) as well as L-arginine x TRIM interaction ($F_{1,48}=4.42$, $P=0.04$) on neuritic length. *Post hoc* analysis revealed that L-arginine-treated neurons had fewer primary neurites ($P<0.05$) and decreased neuritic length ($P<0.01$) when compared to control-treated neurons. Co-treatment with TRIM was capable of reversing the decrease in neuritic length observed with L-arginine treated neurons ($P<0.01$). Two-way ANOVA demonstrated no effects of L-arginine or TRIM on the number of neuritic branches. However,

three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of L-arginine ($F_{1,48}=7.07$, $P=0.01$), TRIM ($F_{1,48}=17.80$, $P<0.001$), L-arginine $\times$ TRIM ($F_{1,48}=4.12$, $P=0.05$), distance ($F_{19,912}=686.38$, $P<0.001$), and TRIM $\times$ distance ($F_{19,912}=7.31$, $P<0.001$) but failed to demonstrate an L-arginine $\times$ TRIM $\times$ distance interaction. *Post hoc* analysis revealed that TRIM increased the number of neuritic branches at 10-40 μm ($P<0.05$). In contrast, L-arginine decreased the number of branches at 10-40 μm ($P<0.01$) and 50 μm ($P<0.05$) when compared to control-treated neurons. TRIM (100 nM) reversed this L-arginine induced decrease at 30 and 40 μm ($P<0.05$) (Figure 3.5B).

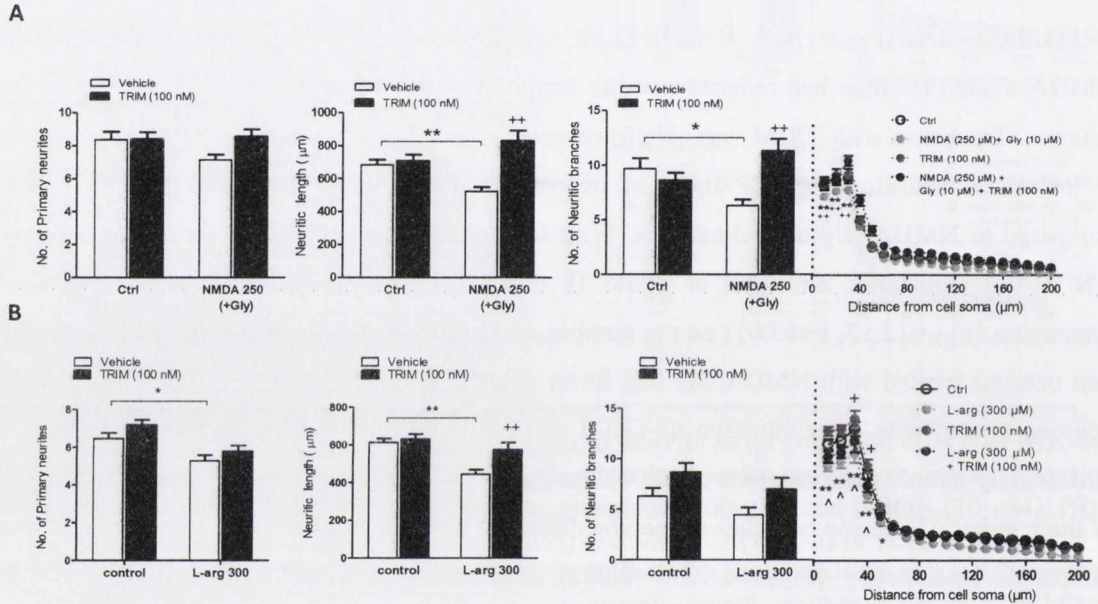


Figure 3.5: Effects of TRIM on NMDA/glycine and L-arginine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of (A) TRIM (100 nM) and NMDA (250 μM)/Gly (10 μM) or (B) TRIM (100 nM) and L-arginine (300 μM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. Data are expressed as mean $\pm$ SEM, $n=16$ -24 coverslips per group representative of 3 independent experiments. ** $P<0.01$, * $P<0.05$ vs. ctrl, ++ $P<0.01$, + $P<0.05$ vs. respective vehicle (Student-Newman-Keuls). Sholl profile, ** $P<0.01$, * $P<0.05$ NMDA250/Gly vs. ctrl and L-arginine (300 μM) vs. ctrl ^ $P<0.05$ TRIM (100 nM) vs. ctrl, ++ $P<0.01$, + $P<0.05$ NMDA250/Gly/TRIM (100 nM) vs. NMDA250/Gly or L-arginine (300 μM)/TRIM (100nM) vs. L-arginine (300 μM) (Student-Newman-Keuls).

3.3.7 Effects of ODQ on L-arginine induced reduction in neurite outgrowth

As soluble guanylate cyclase (sGC) is regarded as the most sensitive physiological receptor for NO, the effect of the sGC inhibitor ODQ (10 μM) was assessed on L-arginine-mediated reductions in neurite outgrowth.

Two-way ANOVA demonstrated an effect of L-arginine ($F_{1,88}=5.60$, $P=0.02$), ODQ ($F_{1,88}=15.10$, $P<0.001$) but failed to demonstrate a L-arginine x ODQ interaction on the number of primary neurites and an effect of L-arginine ($F_{1,80}=8.89$, $P=0.004$), ODQ ($F_{1,80}=10.45$, $P=0.002$), and a L-arginine x ODQ interaction ($F_{1,80}=8.317$, $P=0.005$) on the number of neuritic length. Finally, two-way ANOVA demonstrated an effect of L-arginine ($F_{1,88}=5.60$, $P=0.018$), ODQ ($F_{1,88}=5.56$, $P=0.02$) and a L-arginine x ODQ interaction ($F_{1,88}=10.98$, $P=0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that L-arginine-treated neurons had fewer primary neurites ($P<0.01$), decreased neuritic length ($P<0.01$) and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons. Treatment with ODQ was able to reverse these deficits, as evidenced by increased number of primary neurites ($P<0.01$) neuritic length ($P<0.01$) and number of neuritic branches of neurons treated with L-arginine and ODQ when compared to L-arginine-treated neurons.

Furthermore, three-way repeated measures ANOVA of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of L-arginine ($F_{1,88}=23.71$, $P<0.001$), ODQ ($F_{1,88}=7.79$, $P=0.006$), L-arginine x ODQ interaction ($F_{1,88}=8.32$, $P=0.005$), distance ($F_{19,1672}=1169$, $P<0.001$), L-arginine x distance interaction ($F_{19,1672}=6.86$, $P<0.001$), ODQ x distance interaction ($F_{19,1672}=9.88$, $P<0.001$) and L-arginine x ODQ x distance interaction ($F_{19,1672}=2.453$, $P<0.001$). *Post hoc* analysis revealed that L-arginine decreased the number of branches at 10–40 μm ($P<0.01$) and 50 μm ($P<0.05$) when compared to control-treated neurons. ODQ was able to reverse this decrease at 10–50 μm ($P<0.01$) (**Figure 3.6A, B**).

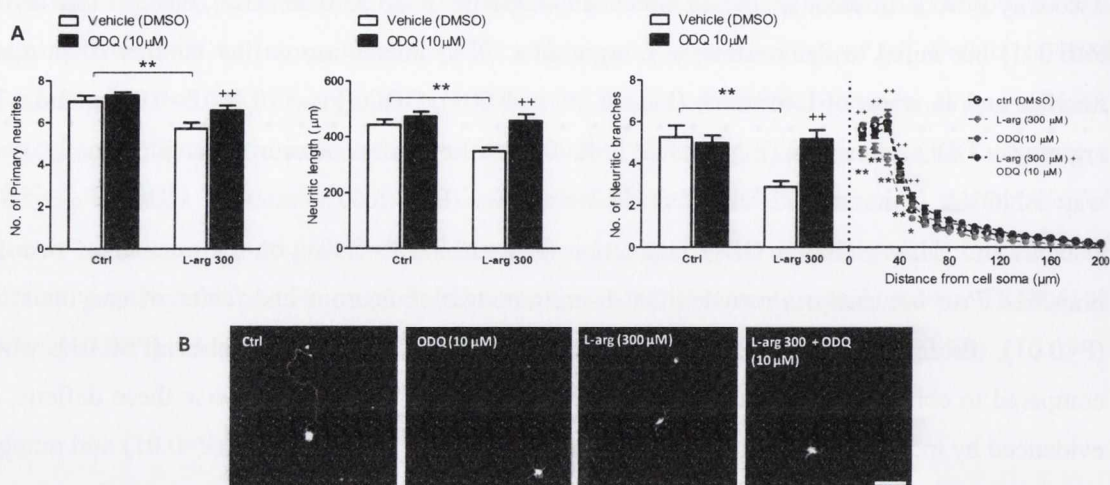


Figure 3.6: Effects of ODQ on L-arginine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of L-arginine (300 μ M) and ODQ (10 μ M). **(A)** Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. **(B)** Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μ m. Data are expressed as mean $\pm$ SEM, $n=22-24$ coverslips per group representative of 3 independent experiments. ** $P<0.01$ vs. ctrl, ++ $P<0.01$ vs. respective vehicle (Student-Newman-Keuls). Sholl profile, ** $P<0.01$, * $P<0.05$ L-arg (300 μ M) vs. ctrl, ++ $P<0.01$, + $P<0.05$ L-arg (300 μ M)/ODQ (10 μ M) vs. L-arg (300 μ M) (Student-Newman-Keuls).

3.3.8 Effects of IC87201 (10 and 100 nM) on NMDA/glycine-induced reduction in neurite outgrowth

Since both ketamine and MK-801 (10 nM) were able to counteract the effects of NMDA-induced reduction in neurite outgrowth, the effects of IC87201 on NMDA/glycine-induced reduction in neurite outgrowth were examined to investigate whether the effects observed with the NMDA-R antagonists generalised to the PSD-95/nNOS inhibitor IC87201.

3.3.8.1 Effects of IC87201 (10 nM) on NMDA/glycine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,130}=4.91$, $P=0.028$) and IC87201 ($F_{1,130}=8.73$, $P=0.0037$) but there was no NMDA/Gly $\times$ IC87201 interaction on the number of primary neurites. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer primary neurites than control-treated neurons ($P<0.01$), which was reversed by treatment with IC87201 ($P<0.01$). Moreover, two-way ANOVA demonstrated an effect of NMDA/Gly on neuritic length and number of branches ($F_{1,130}=6.77$, $P=0.010$ and $F_{1,130}=7.93$, $P=0.0056$, respectively) but failed to demonstrate an IC87201 effect or NMDA/Gly $\times$ IC87201 interaction. *Post hoc* analysis revealed that NMDA-treated neurons had decreased neuritic length ($P<0.05$) and number of

neuritic branches ($P<0.05$) when compared to control-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,130}=24.60$, $P<0.001$), IC87201 ($F_{1,130}=10.22$, $P=0.0017$), NMDA/Gly x IC87201 ($F_{1,130}=6.92$, $P=0.0095$), distance ($F_{19,2470}=2019$, $P<0.001$), NMDA/Gly x distance ($F_{19,2470}=5.94$, $P<0.001$), IC87201 x distance ($F_{19,2470}=5.26$, $P<0.001$) but failed to demonstrate an NMDA/Gly x IC87201 x distance interaction. *Post hoc* analysis revealed that IC87201 increased the number of branches at 20 and 30 μm ($P<0.01$) and 40 μm ($P<0.05$) when compared to control-treated neurons. In contrast, NMDA/Gly decreased the number of branches at 10-30 μm ($P<0.01$) and 50 μm ($P<0.05$) when compared to control-treated neurons. IC87201 reversed this decrease at 10-30 μm ($P<0.01$) and 50 μm ($P<0.05$) (**Figure 3.7A**).

3.3.8.2 Effects of IC87201 (100 nM) on NMDA/glycine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of IC87201 ($F_{1,130}=28.41$, $P<0.001$) and an NMDA/Gly x IC87201 interaction ($F_{1,130}=6.98$, $P=0.0093$) on the number of primary neurites. *Post hoc* analysis revealed that IC87201 increased the number of primary neurites ($P<0.05$), while NMDA/Gly-treated neurons had fewer primary neurites than control-treated neurons ($P<0.01$). IC87201 treatment was able to reverse the NMDA/Gly-induced reduction in primary neurites ($P<0.01$). Moreover, two-way ANOVA demonstrated an effect of IC87201 on neuritic length and number of branches ($F_{1,130}=20.62$, $P<0.001$ and $F_{1,130}=26.17$, $P<0.001$, respectively), an NMDA/Gly x IC87201 interaction on neuritic length ($F_{1,130}=3.74$, $P=0.05$) and an NMDA/Gly x IC87201 interaction on the number of neuritic branches ($F_{1,130}=7.77$, $P=0.0061$). *Post hoc* analysis revealed that NMDA-treated neurons had decreased neuritic length ($P<0.01$) and number of neuritic branches ($P<0.05$) when compared to control-treated neurons that were reversed by IC87201 treatment ($P<0.01$). Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,130}=17.34$, $P<0.001$), IC87201 ($F_{1,130}=69.82$, $P<0.001$), NMDA/Gly x IC87201 ($F_{1,130}=22.85$, $P<0.001$), distance ($F_{19,2470}=2399$, $P<0.001$), NMDA/Gly x distance ($F_{19,2470}=1.68$, $P=0.033$), IC87201 x distance ($F_{19,2470}=22.40$, $P<0.001$) and NMDA/Gly x IC87201 x distance interaction ($F_{19,2470}=7.81$, $P<0.001$). *Post hoc* analysis revealed that IC87201 increased the number of neuritic branches at 10-30 μm ($P<0.01$) and 40 μm ($P<0.05$), while NMDA/Gly decreased the number of branches at 10-30 μm ($P<0.01$) and 50 μm ($P<0.05$) when compared to control-treated neurons. IC87201 reversed the NMDA-induced decrease at 10-30 μm ($P<0.01$) and 50 μm ($P<0.05$) (**Figure 3.7B**).

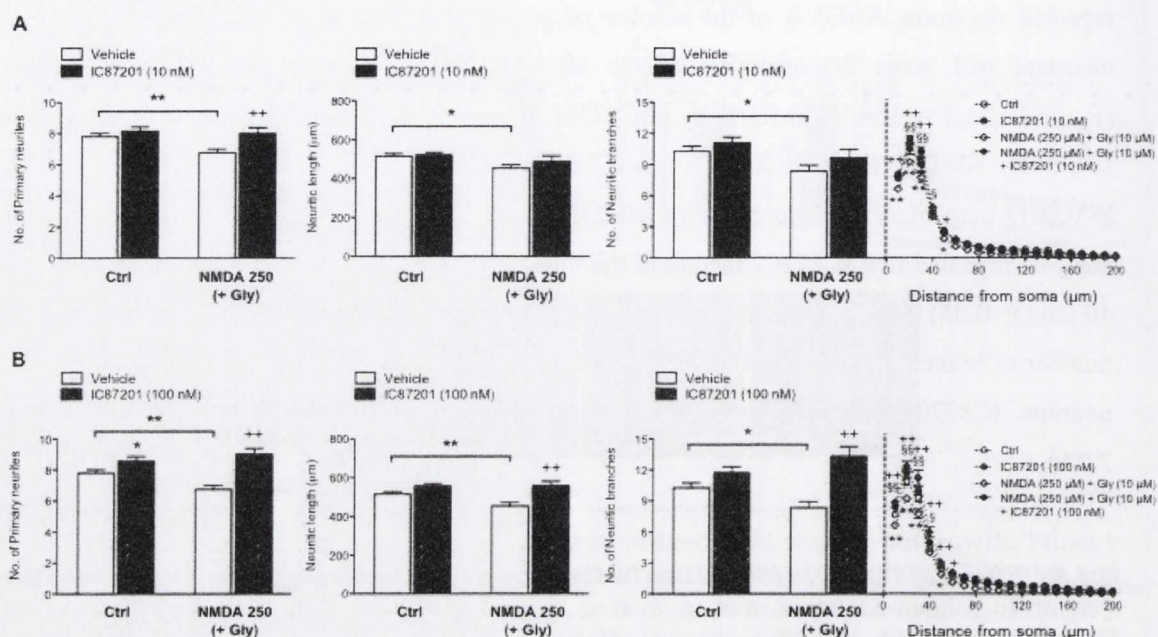


Figure 3.7: Effects of IC87201 on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with (A) a combination of NMDA (250 µM)/glycine (10 µM) and IC87201 (10 nM) or (B) a combination of NMDA (250 µM)/glycine (10 µM) and IC87201 (100 nM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. Data are expressed as mean ± SEM, n=21-39 coverslips per group representative of 3-5 independent experiments. **P<0.01 and *P<0.05 vs. ctrl, ++P<0.01 vs. respective vehicle (Student-Newman-Keuls); Sholl profile, **P<0.01 and *P<0.05 NMDA 250/Gly vs. ctrl, ++P<0.01 and +P<0.05 NMDA 250/Gly/IC87201 vs. NMDA 250/Gly, §§P<0.01 and §P<0.05 IC87201 vs. ctrl (Student-Newman-Keuls).

3.3.9 Effects of ZL006 (10 and 100 nM) on NMDA/glycine-induced reduction in neurite outgrowth

ZL006 (10 and 100 nM) was also investigated for its ability to counteract NMDA/glycine-induced reductions in neurite outgrowth similar to the effects observed for IC87201.

3.3.9.1 Effects of ZL006 (10nM) on NMDA/glycine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,75}=29.58$, $P<0.001$) and ZL006 ($F_{1,75}=4.52$, $P=0.04$) on neuritic length. *Post hoc* analysis revealed that NMDA/Gly treated neurons had reduced neuritic length ($P<0.01$) when compared to control-treated neurons. Treatment with ZL006 reversed this decrease ($P<0.01$). Similarly, two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,75}=31.71$, $P<0.001$) and an effect of ZL006

($F_{1,75}=23.82$, $P<0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA/Gly treated neurons had a decreased number of neuritic branches ($P<0.01$) and ZL006 attenuated this decrease ($P<0.01$). In addition, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,76}=4.48$, $P=0.03$), distance ($F_{19,1444}=46.17$, $P<0.001$), NMDA/Gly x distance ($F_{19,1444}=26.89$, $P<0.001$), ZL006 x distance ($F_{19,1444}=30.54$, $P<0.001$) and NMDA/Gly x ZL006 x distance interaction ($F_{19,1444}=22.66$, $P<0.001$). *Post hoc* analysis revealed that ZL006 increased the number of neuritic branches at 10, 30, 40 and 50 μm ($P<0.01$). In contrast, NMDA/Gly decreased the number of branches at 10, 20 and 50 μm ($P<0.01$) when compared to control-treated neurons. Co-treatment with ZL006 reversed this decrease at 10, 20 and 50 μm ($P<0.01$) (**Figure 3.8A**).

3.3.9.2 Effects of ZL006 (100 nM) on NMDA/glycine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,75}=17.86$, $P<0.001$) and ZL006 (100 nM) ($F_{1,75}=8.55$, $P=0.05$) and a NMDA/Gly x ZL006 interaction ($F_{1,75}=3.69$, $P=0.05$) on neuritic length. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had reduced neuritic length ($P<0.01$) when compared to control-treated neurons. Treatment with ZL006 attenuated this decrease ($P<0.01$). Furthermore, two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,75}=13.48$, $P<0.001$) and an effect of ZL006 ($F_{1,75}=16.84$, $P<0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had a decreased number of neuritic branches ($P<0.01$) and ZL006 reversed this decrease ($P<0.01$). In addition, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of distance ($F_{19,1444}=27.45$, $P<0.001$), NMDA/Gly x distance ($F_{19,1444}=15.32$, $P<0.001$), ZL006 x distance ($F_{19,1444}=21.25$, $P<0.001$) and NMDA/Gly x ZL006 x distance interaction ($F_{19,1444}=17.87$, $P<0.001$). *Post hoc* analysis revealed that ZL006 increased the number of neuritic branches at 30 and 40 μm ($P<0.01$). In contrast, NMDA/Gly decreased the number of branches at 10, 20, 50, 70 and 80 μm ($P<0.01$) when compared to control-treated neurons. Co-treatment with ZL006 reversed this decrease at 50, 70 and 80 μm from the cell soma ($P<0.01$) (**Figure 3.8B**).

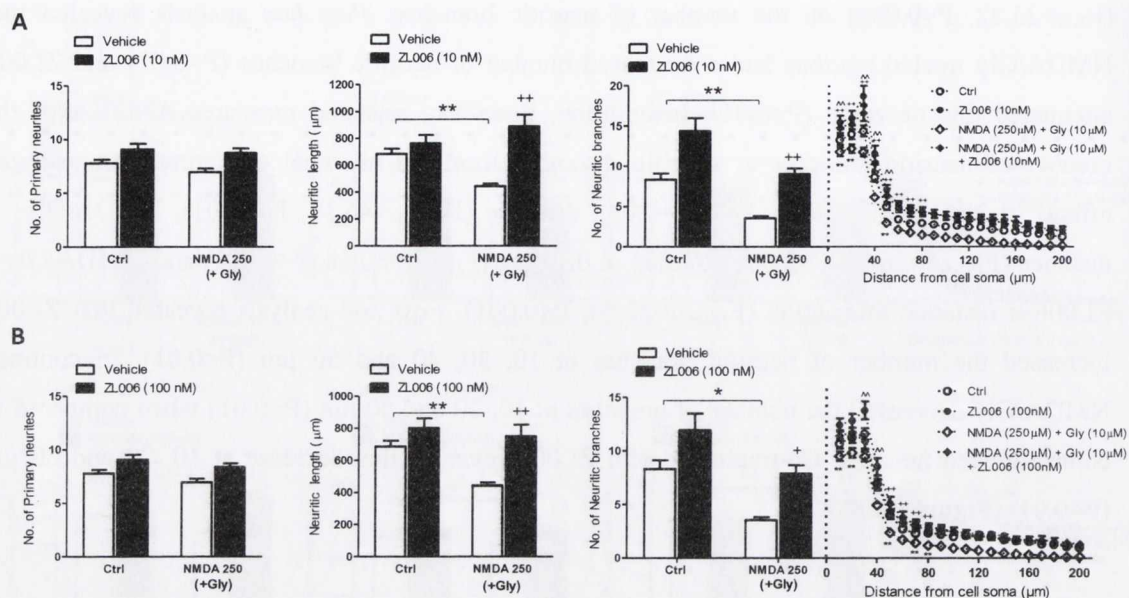


Figure 3.8: Effects of ZL006 on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with (A) a combination of NMDA (250 μ M)/glycine (10 μ M) and ZL006 (10 nM) or (B) a combination of NMDA (250 μ M)/glycine (10 μ M) and ZL006 (100 nM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. Data are expressed as mean $\pm$ SEM, n=20 coverslips per group representative of 3 independent experiments. **P<0.01 and *P<0.05 vs. ctrl, ++P<0.01 vs. respective vehicle (Student-Newman-Keuls); Sholl profile, **P<0.01 and *P<0.05 NMDA 250/Gly vs. ctrl, ++P<0.01 and +P<0.05 NMDA 250/Gly/ZL006 vs. NMDA 250/Gly, ^^P<0.01 and ^P<0.05 ZL006 vs. ctrl (Student-Newman-Keuls).

3.3.10 Effects of IC87201 (100 nM) on L-arginine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of L-arginine ($F_{1,81}=40.34$, $P<0.001$) but failed to show a L-arginine x IC87201 interaction on the number of primary neurites. Two-way ANOVA also showed an effect of L-arginine ($F_{1,83}=16.95$, $P<0.001$), IC87201 ($F_{1,83}=7.11$, $P=0.009$) but failed to demonstrate a L-arginine x IC87201 interaction on the number of neuritic length. Again, two-way ANOVA demonstrated an effect of L-arginine ($F_{1,81}=24.70$, $P<0.001$), IC87201 ($F_{1,81}=2.83$, $P=0.009$) but failed to show a L-arginine x IC87201 interaction on the number of neuritic branches. *Post hoc* analysis revealed that L-arginine-treated neurons had fewer primary neurites ($P<0.01$), decreased neuritic length ($P<0.05$) and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons and treatment with IC87201 failed to reverse these deficits. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of L-arginine ($F_{1,76}=11.68$, $P=0.001$), IC87201 ($F_{1,76}=13.02$, $P<0.001$), distance ($F_{19,1444}=651.04$, $P<0.001$), L-arginine x distance interaction ($F_{19,1444}=2.35$, $P<0.001$), IC87201 x distance interaction ($F_{19,1444}=4.77$, $P<0.001$) but failed to demonstrate a L-arginine x IC87201 x distance interaction. *Post hoc* analysis revealed that IC87201 increased the number of branches at 10-30 μ m when compared to control-treated

neurons. In contrast, L-arginine decreased the number of branches at 10-30 μm ($P<0.01$) and 50 μm ($P<0.05$) when compared to control-treated neurons. (Figure 3.9A, B).

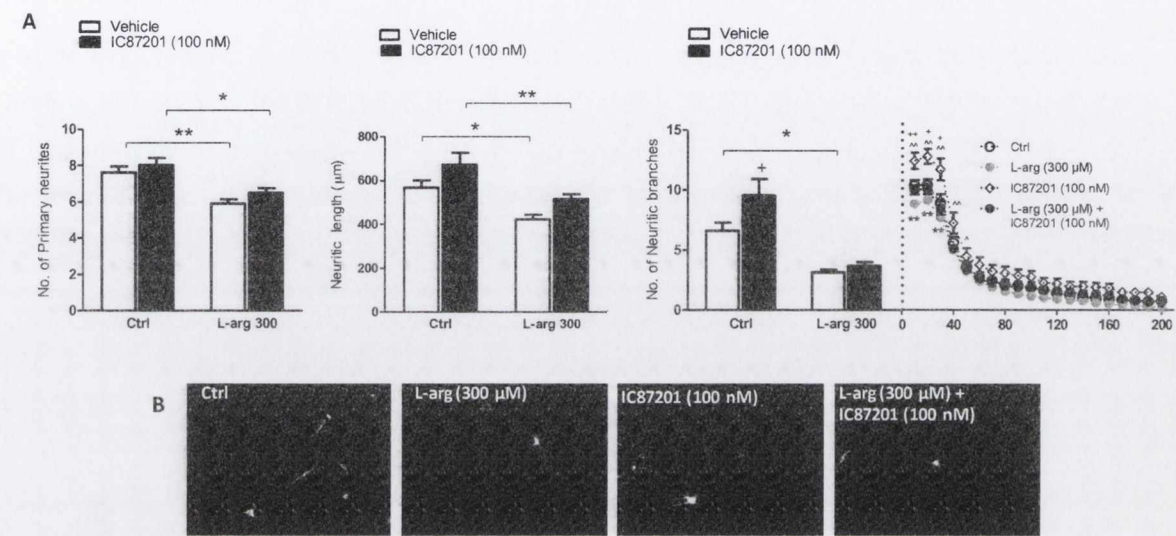


Figure 3.9: Effects of IC87201 on L-arginine-induced reduction in neurite outgrowth.

Primary cortical neurons (DIV 3) were treated for 24 h with a combination of L-arginine (300 μM) and IC87201 (100 nM). (A) Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. (B) Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μm . Data are expressed as mean $\pm$ SEM, n=20-24 coverslips per group representative of 3 independent experiments. *** $P<0.01$, * $P<0.05$ vs. ctrl (Student-Newman-Keuls). Sholl profile, ** $P<0.01$, L-arg (300 μM) vs. ctrl, $^{++}P<0.01$ and $^{+}P<0.05$ IC87201 (100 nM) vs. IC87201 (100 nM) /L-arg (300 μM) $^{\wedge}P<0.01$, $^{\wedge}P<0.05$ IC87201 (100 nM) vs. ctrl (Student-Newman-Keuls).

3.3.11 Concentration-related effects of NMDA on neurite outgrowth of mature (DIV 20) cortical neurons

One-way ANOVA demonstrated a significant effect of NMDA and glycine on the number of primary neurites ($F_{4,58}=13.39$, $P<0.001$), the neuritic length ($F_{4,58}=18.72$, $P<0.001$) and the number of neuritic branches ($F_{4,58}=32.2$, $P<0.001$). *Post hoc* analysis revealed that NMDA (50 - 250 μM) combined with glycine (10 μM) decreased the number of primary neurites ($P<0.001$), the neuritic length ($P<0.001$) and the number of neuritic branches ($P<0.001$) when compared to control-treated neurons. In contrast, NMDA at 10 μM had no effect on neurite outgrowth compared to control-treated neurons (**Figure 3.10**).

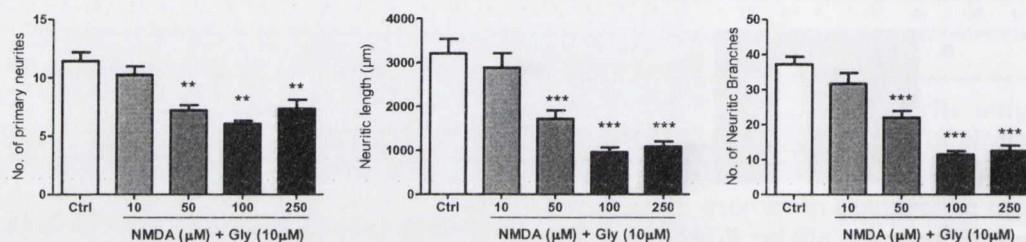


Figure 3.10: Concentration-related effects of NMDA/glycine on neurite outgrowth of mature (DIV20) cortical neurons. Primary cortical neurons (DIV 20) were treated for 24 h with NMDA (10 - 250 μM)/glycine (10 μM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length, number of branches and Sholl profile. Data are expressed as mean $\pm$ SEM, $n=12-15$ coverslips per group representative of 3 independent experiments. *** $P<0.001$ vs. Ctrl (Student-Newman-Keuls).

3.3.12 Effects of MK-801 and ketamine on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,136}=5.7$, $P=0.02$), an effect of MK-801 ($F_{1,136}=17.16$, $P<0.001$) and a NMDA/Gly $\times$ MK-801 interaction ($F_{1,136}=8.38$, $P=0.004$) on the co-localised expression of synaptic markers. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised synaptophysin and PSD-95 synaptic puncta than control neurons. Co-treatment with MK-801 protected against these deficits ($P<0.01$) (**Figure 3.11A, B**).

A two-way ANOVA also demonstrated an effect of ketamine ($F_{1,129}=14.14$, $P<0.001$) and a NMDA/Gly $\times$ ketamine interaction ($F_{1,129}=6.65$, $P=0.01$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons

had fewer co-localised synaptophysin and PSD-95 puncta than control neurons and co-treatment with ketamine protected against these effects ($P<0.01$) (**Figure 3.11C**).

3.3.14 Effects of TRIM on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,134}=9.58$, $P=0.002$) and a NMDA/Gly x TRIM interaction ($F_{1,134}=13.09$, $P<0.001$) on the co-localised expression of synaptic markers. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised synaptophysin and PSD-95 puncta than control-treated neurons and co-treatment with TRIM attenuated these NMDA/Gly-induced effects ($P<0.01$) (**Figure 3.11D**).

3.3.15 Effects of TRIM on L-arginine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of L-arginine ($F_{1,105}=4.39$, $P=0.04$) and a L-arginine x TRIM interaction ($F_{1,105}=9.19$, $P=0.003$) on the co-localised expression of synaptic markers. *Post hoc* analysis revealed that L-arginine-treated neurons had fewer co-localised synaptophysin and PSD-95 synaptic puncta than control-treated neurons. Co-treatment with TRIM protected against the L-arginine-induced effects ($P<0.05$) (**Figure 3.11E**).

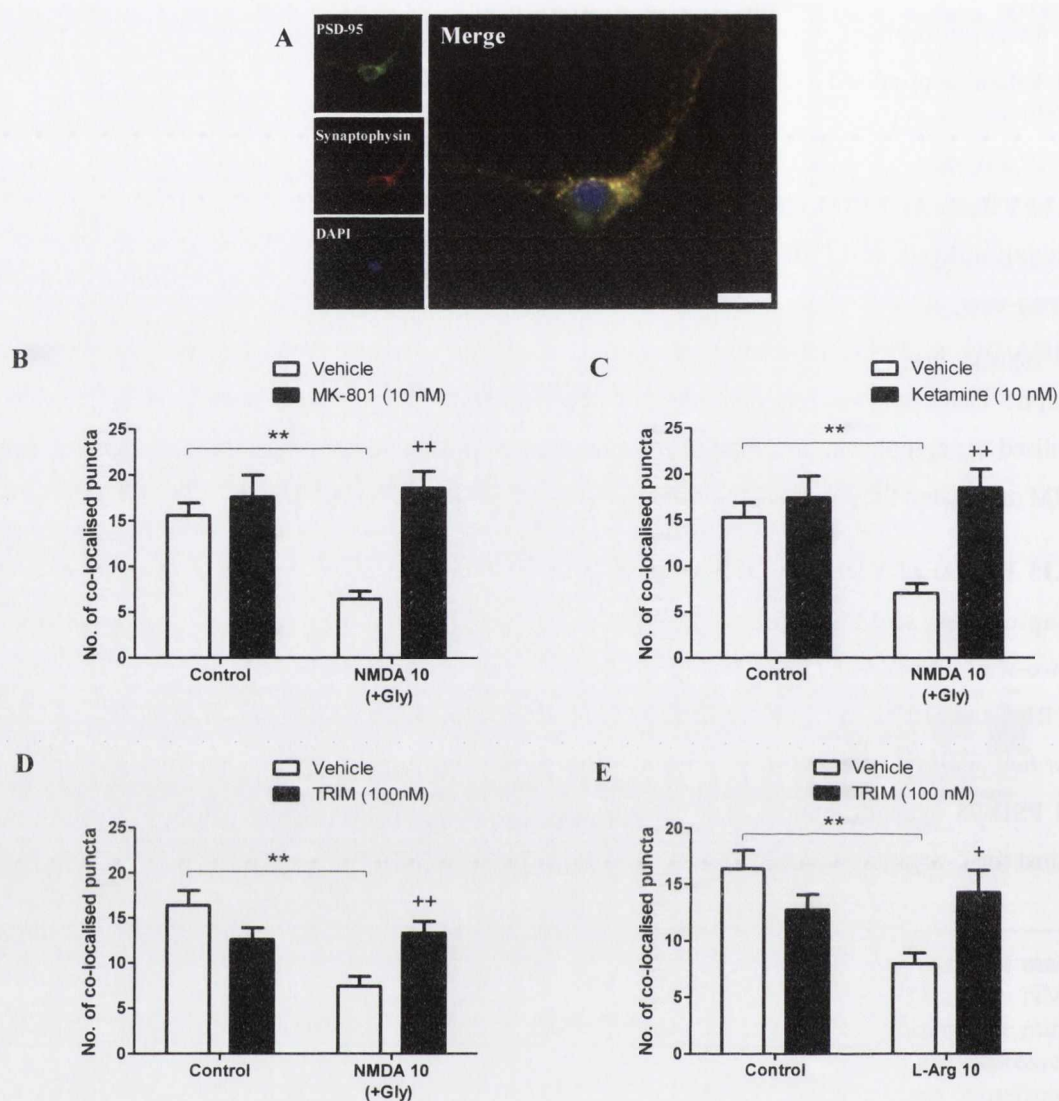


Figure 3.11: Effects of NMDA-R antagonism and nNOS inhibition on NMDA/glycine- or L-arginine-induced reduction in the co-localised expression of synaptophysin and PSD-95

(A) Primary cortical neurons (DIV 21) were stained using the post-synaptic marker PSD-95 (green), the pre-synaptic marker synaptophysin (red) and the DAPI nuclear stain (blue). Quantification of co-localised puncta was conducted using ImageJ software. Scale Bar = 20 μ m. Neurons (DIV 20) were treated for 24 h with NMDA (10 μ M)/Gly (10 μ M) in combination with MK-801 (10 nM) (B), ketamine (10 nM) (C) or TRIM (100 nM) (D). Neurons (DIV 20) were also treated for 24 h with L-arg (10 μ M) in combination with TRIM (100 nM) (E). Data are expressed as mean $\pm$ SEM, n=30-40 neurons per group representative of 3 independent experiments. **P<0.01 vs. Control, ++P<0.01, +P<0.05 vs. respective vehicle (Student-Newman-Keuls).

3.3.15 Effects of ZL006 and IC87201 on NMDA-induced reduction in the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,153}=21.26$, $P<0.001$) and an effect of ZL006 ($F_{1,153}=15.31$, $P<0.001$) on the co-localised expression of synaptic markers.. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised synaptophysin and PSD-95 puncta than control neurons and that co-treatment with ZL006 protected against these effects ($P<0.01$) (**Figure 3.12A**).

A two-way ANOVA also demonstrated an effect of NMDA/Gly ($F_{1,108}=10.39$, $P=0.002$) and an effect of IC87201 ($F_{1,108}=6.37$, $P=0.01$) and a NMDA/Gly x IC87201 interaction ($F_{1,108}=14.08$, $P<0.001$) on the co-localised expression of synaptic markers. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised synaptophysin and PSD-95 puncta than control neurons and that co-treatment with IC87201 attenuated this decrease ($P<0.01$) (**Figure 3.12B**).

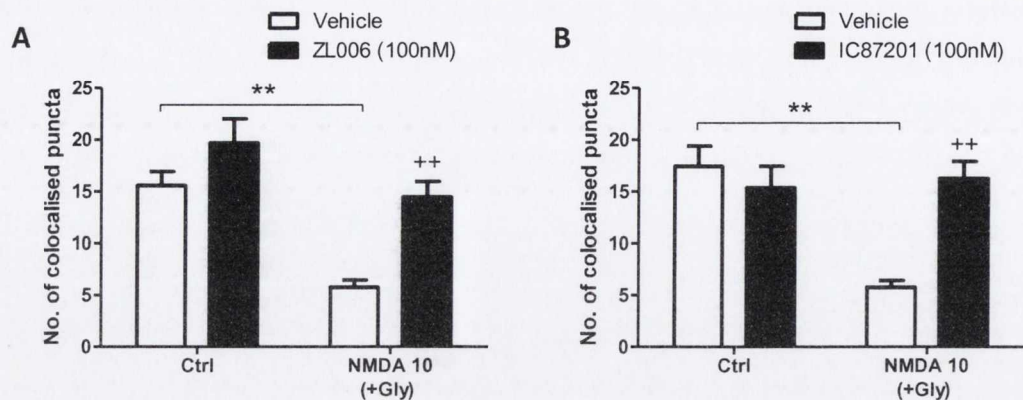


Figure 3.12: Effects of ZL006 and IC87201 on NMDA/glycine induced reduction in the co-localised expression of synaptophysin and PSD-95. Primary cortical neurons (DIV 20) were treated for 24 h with a combination of NMDA (10 μ M)/Gly (10 μ M) and **(A)** ZL006 (100 nM) or **(B)** IC87201 (100 nM). Neurons were fixed and stained with the pre-synaptic marker, synaptophysin, and the post-synaptic marker, PSD-95 and quantification of co-localised synaptic puncta was conducted using ImageJ software. Data are expressed as mean $\pm$ SEM, $n=30-40$ neurons per group representative of 3-4 independent experiments. ** $P<0.01$ vs. ctrl, ** $P<0.01$, vs. respective vehicle (Student-Newman-Keuls).

3.4. Discussion

The results of this investigation demonstrated that the treatment of cultured primary cortical rat neurons (DIV 3) for 24 h with glutamate (500 μ M) or NMDA (100-500 μ M) combined with glycine suppressed neurite outgrowth. A similar reduction of neurite outgrowth was observed with the NO precursor, L-arginine, and the NO donor, SNP (100 and 300 μ M). In more mature neuronal cultures (DIV 20), the results demonstrated that NMDA (10 μ M) and L-arginine (10 μ M) decreased the co-localised expression of pre-synaptic synaptophysin and post-synaptic PSD-95 in synaptic puncta, indicating a decrease in synapse number (Ippolito and Eroglu, 2010, Roqué et al., 2014). The NMDA-R antagonists ketamine (10 nM) and MK-801 (10 nM) counteracted the NMDA/glycine-induced reduction in neurite outgrowth and co-localised expression of synaptic proteins, and the nNOS inhibitor TRIM (100 nM) counteracted both the NMDA/glycine- and L-arginine-induced decreases in neurite outgrowth and synaptic protein co-localisation. Furthermore, inhibition of the main NO receptor, sGC, using the selective inhibitor, ODQ (10 μ M), also protected against L-arginine-induced decreases in neurite outgrowth. Of particular interest, the data presented here demonstrated that treatment with small-molecule inhibitors of the PSD-95/nNOS interface 2-((1H-benzo[d] [1,2,3]triazol-5-ylamino) methyl)-4,6-dichlorophenol (IC87201) (10-100 nM) and 4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid (ZL-006) (10-100 nM) attenuated NMDA/glycine-induced decreases in neurite outgrowth and decreases in synaptic protein co-localisations. These data support the hypothesis that targeting the NMDA-R/PSD-95/nNOS interaction downstream of NMDA-R promotes neurotrophic effects by preventing neurite shrinkage and synapse loss in response to glutamatergic stimulation. The PSD-95/nNOS interface may be an attractive target for treating deficits in neuronal outgrowth and atrophy associated with excessive glutamatergic neurotransmission in neurodevelopmental and neurodegenerative conditions.

3.4.1 Biphasic effects of glutamate on neurite outgrowth

The results of the present investigation demonstrated that glutamate treatment (100 μ M) increased the number of neuritic branches and neuritic length, while higher concentrations (500 μ M) did not influence any of these parameters. These results are in contrast with previous reports which have demonstrated reduced dendritic growth following treatment with glutamate (100 μ M) (Monnerie et al., 2003). We investigated the biphasic effects of glutamate on neurite outgrowth in further detail and hypothesised that the neurotrophic effects observed with glutamate at 100 μ M would mainly be mediated by AMPA-R stimulation while higher concentrations of glutamate (500 μ M) would stimulate both AMPA-R and NMDA-R. To test this hypothesis, cortical cultures were treated with glutamate and glycine, the co-agonist of glutamate that acts as a facilitator for NMDA-R activation (Dingledine et al., 1999) or treated with the AMPA-R antagonist, NBQX, in

the presence of glutamate (100 μ M). The increase in neurite outgrowth observed with glutamate (100 μ M) was abolished by the addition of glycine or co-treatment with NBQX (10 μ M), suggesting that NMDA-R and AMPA-R activation play distinct roles in glutamate mediated neurite outgrowth responses.

3.4.2 NMDA induces neurite atrophy

Although the deleterious effects of glutamate on neurite outgrowth have been described in prior reports, (Mattson et al., 1988, Metzger et al., 1998, Wilson and Keith, 1998, O'Brien et al., 2000, Sunico et al., 2010), the role of the NMDA-R in producing these effects has not been examined in as much detail (Esquenazi et al., 2002, Monnerie et al., 2003, Podestá et al., 2014). In this regard, the results of the present investigation showed that NMDA (250 and 500 μ M) in the presence of glycine (10 μ M) reduced the number of neuritic branches and neuritic length of neurons in immature cortical neurons (DIV 3). In more mature (DIV 20) neuronal cultures, NMDA at lower concentrations (50 - 250 μ M) in the presence of glycine (10 μ M) dose-dependently decreased the number of primary neurites, neuritic length and number of neuritic branches, which is in line with the higher sensitivity of more mature neurons to glutamatergic stimuli (Monnerie et al., 2003). Following on from these effects, we demonstrated that the NMDA-R antagonist MK-801 (10 nM) protected against NMDA/glycine-induced decreases in neuritic length and neuritic branches in immature cortical neurons. Similarly, ketamine (10 nM) protected against NMDA/glycine-induced deficits in the number of primary neurites extending from the cell soma, the number of branches and neuritic length. In addition, both MK-801 (10 nM) and ketamine (10 nM) prevented the reductions in the co-localised expression of synaptophysin and PSD-95 in synaptic puncta induced by NMDA (10 μ M) and glycine (10 μ M) in neurons at DIV 20. Together, these data suggest that the deleterious effects of glutamate on neurite outgrowth and synapse loss are mediated by the NMDA-R. These results are of interest in relation to research indicating the role of glutamatergic stimulation on neurite outgrowth and synapse number in disease pathology. For instance, synapse loss is a prominent pathological feature of Alzheimer's disease and prior *in vitro* research has shown that NR2B containing NMDA-Rs contribute to A β oligomer-induced synapse loss in neuronal cultures and demonstrate that the selective NR2B antagonists, ifenprodil and Ro 25-6981, block these synaptic deficits (Rönicke et al., 2011). Furthermore, chronic stress has been shown to reduce synapse and spine density in several brain regions in animal models of depression, and the antidepressant-like effects observed following ketamine administration in these models is believed to be associated with an increase in the production of synaptic proteins and a promotion of spine and synapse formation in the PFC (Li et al., 2010).

3.4.3 Increasing NO concentration induces neurite atrophy

Considering the functional association between the NMDA-R and nNOS, the effects of raised NO concentrations on neurite outgrowth responses were investigated. Previous reports have demonstrated that the NO donors sodium nitrite (100 μ M) and SNP (100 nM) enhance neurotrophin-induced neurite outgrowth in hippocampal neurons grown in the presence of astrocytes (Hindley et al., 1997). Other studies have indicated that treatment with higher concentrations of SNP (μ M - mM range) mimic the cytotoxic effect of NMDA on neuronal cultures (Dawson et al., 1991, Dawson et al., 1993) and evidence also exists to suggest that NO donors 3-morpholino-sydononimine (SIN-1) (1-2 mM) and S-nitrosocysteine (0.4-1 mM) inhibit the growth of dorsal root ganglion (DRG) neurons (Hess et al., 1993). The data presented in the current investigation are in line with these latter reports and we demonstrated that both the NO donor, SNP (300 μ M), and the NO precursor, L-arginine (300 μ M), reduced the number of primary neurites extending from the cell soma, the neuritic length and the number of neuritic branches at several points from the soma (10 - 200 μ m for SNP and 10-30 μ m for L-arginine). SNP at lower concentrations (30 μ M and 100 μ M) also reduced the neuritic length, and SNP (100 μ M) decreased the number of neuritic branches at 150-180 μ m from the cell soma. Treatment with TRIM (100 nM) was capable of reversing NMDA-induced reductions in neuritic length and branching at 10-30 μ m from the cell soma, and also protected against NMDA/glycine-induced reductions in synaptic protein co-localisation, thus indicating that NMDA/glycine-induced atrophy are associated with increased levels of NO production from NMDA-R coupled nNOS. TRIM (100 nM) also reversed L-arginine-mediated neurite retraction, specifically in terms of the number of primary neurites, neuritic length and branching points at 30 μ m and 40 μ m from the cell body. In more mature neuronal cultures, we demonstrated that L-arginine (10 μ M) decreased the co-localisation of synaptophysin and PSD-95, suggesting a decrease in synapse number following treatment with L-arginine, and we demonstrated that TRIM (100 nM) protected against these synaptic deficits.

3.4.4 sGC plays a role in NO-induced atrophy

The current study also investigated whether decreases in neurite outgrowth induced by NO involved activation of the main NO receptor, sGC, by testing the ability of the sGC inhibitor, ODQ, to protect against L-arginine-induced reductions in outgrowth. We demonstrated that treatment of neurons with ODQ (10 μ M) protects against L-arginine-mediated decreases in the number of primary neurites, the neuritic length and number of branches at 10 - 50 μ m from the cell soma. sGC is responsible for the conversion of GTP to cGMP, and is regarded as the most sensitive receptor for NO (Garthwaite and Boulton, 1995). Basal activity of sGC appears to be an important regulator of neurite development and neuritogenesis (Shelly et al., 2010). In line with this, previous reports have shown that treatment with ODQ inhibits neurite outgrowth induced by

NGF in PC12 cells (Yamazaki et al., 2004, Gigliucci et al., 2010). However, inhibition of sGC has been reported to offer neuroprotection against the effects of excess NO, with reports indicating that ODQ attenuates nitroprusside-induced accumulation of cGMP in rat superior cervical ganglia (Southam et al., 1996) and prevents cell death induced by both glutamate and NO donor (SNAP)-induced neuronal death in primary cultures of cerebellar neurons (Montoliu et al., 1999). The results presented here are in agreement with these reports, and demonstrate that inhibition of sGC provides protection against L-arginine-induced neurite outgrowth responses, thus implicating a role for sGC in mediating the deleterious effects of NO on neurite outgrowth. Interestingly, prior research has reported that NO induces neurite retraction and potential synaptic loss in motoneurons through a mechanism involving cGMP/protein kinase G (PKG) pathway/Rho kinase dependent mechanism, leading to actin depolymerisation and synapse elimination (Sunico et al., 2010). These results provide a putative mechanism linking increased NO levels to actin destabilisation via Rho kinase signalling, and suggests that NO may play a key role in neuronal and synapse loss in multiple neuropathological conditions (Moreno-López et al., 2011).

3.4.5 Small-molecule inhibitors of the PSD-95/nNOS interaction protect against NMDA-induced atrophy

Of particular interest in this study were the effects of two small-molecule inhibitors of the PSD-95/nNOS interaction, namely IC87201 and ZL006, on NMDA-induced neuronal atrophy and synapse loss. The present data indicated that treatment with both IC87201 (10 and 100 nM) and ZL006 (10 and 100 nM) were capable of protecting against NMDA/glycine-induced reductions in neurite outgrowth in neurons at DIV 3. More specifically, IC87201 (10 nM) reversed the NMDA/glycine-induced reduction in primary neurites and proximal branches (10-30 μ m), while a higher concentration of IC87201 (100 nM) blocked each of the NMDA/glycine-induced reductions in neurite output (number of primary neurites, number of branches, neuritic length). In addition, ZL006 (10 nM and 100 nM) reversed NMDA/glycine-induced decreases in the neuritic length and the number of primary neurites. The results presented in the current investigation also demonstrated that IC87201 (100 nM) and ZL006 (100 nM) protected against NMDA-induced reductions in synaptic protein co-localisations in neurons at DIV 20.

An increasing body of literature is reporting beneficial effects of IC87201 and ZL006 on disease models *in vitro* and *in vivo*. Early research reported that IC87201 reduced NMDA-induced cGMP production in rat hippocampal neurons with an IC_{50} of 2.7 μ M (Florio et al., 2009). In addition, plasma levels of IC87201 following intraperitoneal (i.p.) administration of IC87201 (1 mg/kg) in mice peaked 15 min following administration, reaching 55 ng/mL (0.2 μ M), concurrent with anti-nociceptive effects. When administered intrathecally, the *in vivo* potency of IC87201 in the mouse

NMDA-induced thermal hyperalgesia model was 1 pmol (or 0.2 μ M) at the injection site (Florio et al., 2009). In culture, the effects of IC87201 on neurite outgrowth were observed over a similar range of concentrations to those required to reverse NMDA-induced hypersensitisation in mice. The effects of ZL006 on neurite outgrowth were greatest at 100 nM, which corresponds to the IC₅₀ of this compound to inhibit the binding of nNOS to PSD-95 in cortical neurons following stimulation of mature cortical neurons with a combination of glutamate (50 μ M) and glycine (10 μ M) for 30 min (Zhou et al., 2010). Other recent data indicates that intravenous ZL006 (1.5mg/kg/d) enhances neuronal survival and promotes migration of new neurons into the injury area after MCAO-induced ischemia in rats, and increases neurite outgrowth in newborn neurons and dendritic spine formation in mature neurons in the ischemic brain (Luo et al., 2014). The authors also report neurogenic and neurotrophic effects of ZL006 (10 μ M) in co-cultures of neurons with neural stem cells (NSCs) in the oxygen and glucose deprivation (OGD) model of ischemia, lending further support to the neurotrophic properties of this compound (Luo et al., 2014). Both IC87201 and ZL006 have also demonstrated neuroprotective effects against MPP⁺ induced neuronal injury and apoptotic cell death in an *in vitro* model of Parkinson's Disease consisting of cortical neurons at DIV 8-10 (Hu et al., 2014). IC87201 was capable of attenuating MPP⁺ induced neuronal injury at a concentration of 100 nM, while higher concentrations (1 and 10 μ M) of IC87201 and ZL006 also protected against these effects. The higher concentrations required in these studies may reflect the nature of the neuronal insult (OGD-induced reductions in neurite growth or MPP⁺-induced mitochondrial dysfunction rather than NMDA-induced neurite regression) as well as the type of culture (purified neurons vs. cocultures) and the level of maturation of the neurons under investigation.

3.4.6 IC87201 fails to protect against L-arginine-induced atrophy

Finally, to test the specificity of small molecule inhibitors of PSD-95/nNOS, we assessed the ability of IC87201 to prevent reductions in neurite outgrowth induced by downstream L-arginine mediated increases in NO levels. We demonstrated that the L-arginine-induced neurite reduction cannot be abolished by co-treatment with IC87201 (100 nM). Thus, IC87201 selectively protects against NMDA/glycine-evoked reductions in neurite outgrowth. These results help to confirm that IC87201 does not affect nNOS catalytic activity and also indicate that it acts specifically through uncoupling the PSD-95/nNOS protein complex, as the detrimental effects of downstream NO production by L-arginine cannot be attenuated by IC87201.

3.4.7 Conclusions

The results of this investigation establish a role for the NMDA-R/PSD-95/nNOS protein complex in neurite outgrowth and in the co-localisation of synaptic markers, which is taken as a measure of synapse number. We demonstrate a particular role for the NMDA-R in mediating the detrimental effects of glutamatergic stimulation in neuronal atrophy and potential synapse loss and we show

that NMDA-R antagonists, MK-801 and ketamine, protect against these effects. Further downstream, we show that NO induces neuronal atrophy and reductions in co-localised synaptic proteins, and we demonstrate that TRIM protect against this NMDA- and NO-induced neuronal atrophy. We provide novel data which demonstrates that small molecule inhibitors of the PSD-95/nNOS interaction abolish neuronal atrophy induced by NMDA, and restore neuronal growth and co-localised synaptic protein puncta to basal levels following NMDA-R stimulation. These findings suggest that inhibition of the PSD-95/nNOS protein interaction may represent a promising target for the development of therapeutics aimed at providing neurotrophic effects and promoting neuronal remodelling in CNS pathologies related to neuronal atrophy and synapse loss.

Chapter 4: Peptide-mediated inhibition of the NOS1AP/nNOS interface protects against NMDA-induced atrophy in primary cortical neurons

4. 1 Introduction

Nitric oxide synthase adaptor protein 1 (NOS1AP), also known as CAPON (C-terminal PDZ ligand of neuronal nitric oxide synthase), is an adaptor protein which binds to nNOS and plays a role in mediating NMDA-R and NO signalling, as well as linking nNOS to effector proteins such as Dexras and synapsin (Courtney et al., 2014, Li et al., 2015). Alterations in the *nos1ap* gene and increased NOS1AP protein levels have been reported in various neurological disorders, most notably neuropsychiatric illnesses including schizophrenia, anxiety and autism (Xu et al., 2005, Brzustowicz, 2008, Freudenberg et al., 2015), but also in sudden cardiac death and long QT syndromes (Crotti et al., 2009, Kao et al., 2009, Kapoor et al., 2014). This evidence has led to speculation that targeting NOS1AP may provide useful therapeutic properties in these disease states. In addition, given that NOS1AP is a downstream component of NMDA-R/PSD-95/nNOS signalling, is it also of interest as a pharmacological site for the development of drugs aimed at treating disorders associated with excitotoxicity and abnormal glutamate signalling such as ischaemia, major depression and neuropathic pain (Li et al., 2015).

Neuronal atrophy and aberrant neuronal connectivity patterns are prominent pathological features of illnesses associated with NOS1AP disturbances. Notably, schizophrenia – in which *nos1ap* is a genetic risk factor (Brzustowicz, 2008, Hadzimichalis et al., 2010, Freudenberg et al., 2015) – is increasingly regarded as a neurodevelopmental disorder involving neuronal connectivity disturbances occurring during embryonic and early postnatal development which manifest in behavioural and cognitive symptoms later in life. Furthermore, neuronal atrophy and decreased spine and synapse number have also been reported in several brain regions in illnesses associated with glutamatergic hyperactivity such as major depression, stroke and neurodegenerative disorders (Duman et al., 2012, Sanacora et al., 2012). Since NMDA-R antagonism and nNOS inhibition are both associated with adverse and off-target side effects, recent research is aimed at targeting specific protein interactions and signalling pathways downstream of NMDA-R coupled to nNOS (Cao et al., 2005, Vaslin et al., 2011). The NOS1AP/nNOS protein interaction and its downstream signalling is one such target.

At least two isoforms of NOS1AP have been described in humans – a full length, long (NOS1AP-L) isoform which is a 506 aa protein encoded by the expression of all 10 exons of the *nos1ap* gene, and a short (NOS1AP-S) isoform consisting of a 210 aa protein generated from the expression of only the last 2 exons of the gene. NOS1AP-L contains two main functional domains – an N terminal phosphotyrosine-binding (PTB) domain as well as the C-terminal PDZ binding motif (Xu et al., 2005, Brzustowicz, 2008, Carrel et al., 2009). The PDZ domain of nNOS binds to NOS1AP, and NOS1AP was initially regarded as an inhibitor of nNOS by competing with PSD-95 for binding to the PDZ domain of nNOS, and was therefore presumed to reduce NMDA-

R signalling (Jaffrey et al., 1998). However, more recent functional studies suggest that NOS1AP may act to mediate rather than inhibit NMDA-R/PSD-95/NO signalling. Although the nature of the NOS1AP and nNOS interaction is not fully understood, recent research identifies two sites of interaction between the NOS1AP and the nNOS PDZ domain. The first is a recently identified site of NOS1AP between amino acid 400-431 which binds nNOS with high affinity, referred to as the ExF motif, and the second is the PDZ motif (residues 488-506) of NOS1AP which binds nNOS with lower affinity and is thought to play an important role in stabilising the interaction between the two proteins (Li et al., 2015).

Multiple lines of evidence suggest NOS1AP plays a key role in various aspects of neuronal development including dendrite patterning and outgrowth (Carrel et al., 2009), cortical neuronal migration (Carrel et al., 2015) and synapse and spine development (Richier et al., 2010). Overexpression of NOS1AP-L has been reported to decrease dendritic branching of cultured hippocampal neurons between days *in vitro* (DIV) 0-12, with these effects being linked to a region of NOS1AP between amino acids 181-307 which bind carboxypeptidase E (CPE) (Carrel et al., 2009). In terms of spine and synapse development, NOS1AP overexpression has been found to increase dendritic protrusions, an effect which is dependent on NOS1AP interacting with the membrane protein, Scribble, via the phosphotyrosine binding (PTB) domain of NOS1AP (Richier et al., 2010). Furthermore, *in vivo* overexpression of NOS1AP has recently been reported to disrupt cortical neuronal migration and decrease dendritogenesis, effects that were also shown to be mediated through the PTB domain of NOS1AP (Carrel et al., 2015). These results suggest NOS1AP is an important regulator of dendrite morphology independent to its role in binding nNOS via the PDZ domain and regulating NO signalling.

Excitotoxic stimulation of neuronal cells with NMDA has previously been shown to recruit NOS1AP to nNOS, and this interaction has been reported to lead to p38 MAPK activation and the recruitment of cell death pathways (Li et al., 2013). In the current investigation, we employed the use of trans-activating transcriptional activator (TAT) fused cell penetrating peptides (CPPs) to investigate the role of the NOS1AP/nNOS protein interaction in changes to neurite outgrowth and synaptic protein co-localisations as a measure of synapse number following the application of NMDA in primary cortical neurons *in vitro*. Inhibition of protein-protein interactions through the use of synthetic CPPs is regarded as an attractive method of inhibition compared to direct receptor antagonism or enzyme inhibition. This is mainly due to the fact that CPPs are capable of specifically uncoupling two proteins from one another, allowing the modification of a particular signalling pathway without disturbing other functions of the proteins. CPPs are short basic peptides that can cross cell membranes and can be used to transport various cargoes into cells for specific purposes including peptide inhibition of protein-protein interactions (Vaslin et al., 2011). This type of inhibition is achieved through the delivery of excess amounts of the amino acid

sequence of one of the proteins which is needed for the interaction, linked to transporters such as the commonly used 10 amino acid HIV TAT(48-57) transporter sequence, which confers cell permeability to the cargo by virtue of its short length and efficacy of cell membrane translocation (Borsello and Bonny, 2004). Inhibition of the NOS1AP/nNOS interaction has been successfully achieved in this way by using a peptide termed L-TAT-GESV. This peptide acts by competing with the PDZ domain of nNOS that interacts with NOS1AP, and was found to inhibit NMDA-induced recruitment of NOS1AP to nNOS and provide neuroprotection in neuronal cultures and *in vivo* in a model of neonatal hypoxia-induced ischaemia (Li et al., 2013).

Overactivation of NMDA and excitotoxicity are well known to activate the stress related protein kinases p38 and JNK, and activation of these kinases are believed to be involved in excitotoxic cell death mechanisms (Cao et al., 2005, Centeno et al., 2007, Molz et al., 2008, Soriano et al., 2008, Yang et al., 2012). In support of this, both p38 MAPK inhibitors and JNK inhibitors have been reported to offer neuroprotection in cell culture models of excitotoxicity as well as in animal models of ischaemia (Borsello et al., 2003, Piao et al., 2003). NOS1AP is known to interact with the p38 activating kinase, MKK3, and L-TAT-GESV was shown to be capable of reducing p38 MAPK activation (Li et al., 2013), thus linking activation of the NOS1AP/nNOS interaction to p38 activation and cell death pathways. A promising protease resistant peptide inhibitor of JNK, D-JNKI-1, has also been shown to offer neuroprotection in animal models of cerebral ischaemia (Borsello et al., 2003). This peptide acts by competitively preventing JNK from interacting with its main target, c-Jun. The peptide was developed by linking the HIV TAT(48-57) transporter sequence to the 20 amino acid JNK binding motif of JNK-interacting protein-1/islet-brain 1 (JIP-1/IB1). The binding motif of JIP-1/IB1 is similar to that of c-Jun except that JNKs affinity for binding to JIP-1/IB1 is reported to be 100 fold higher than that of its affinity for c-Jun (Bonny et al., 2001, Borsello et al., 2003).

In this study, we investigated the role of the NOS1AP/nNOS interaction in neurite outgrowth and synapse number in primary cortical neurons treated with NMDA. We employed the use of L-TAT-GESV to assess whether selectively uncoupling NOS1AP from nNOS can provide neuroprotection against NMDA-induced reductions in neurite outgrowth and reductions in synaptic protein co-localisations. Since NMDA and NOS1AP signalling have been linked to the activation of p38 MAPK pathway (Li et al., 2013), we also investigated the potential role of this kinase in neurite outgrowth and synaptic protein co-localisation by firstly assessing the effects of the commercially available p38 MAPK inhibitors, SB203580 and BIRB796, on NMDA-induced decreases in outgrowth and synaptic protein co-localisation. Following on from this, we also investigated the effects of an experimental TAT-fused peptide p38 MAPK inhibitor, TAT-D1, which acts by inhibiting the recruitment of the p38 MAPK pathway by NOS1AP. Finally, we

assessed whether JNK inhibition using the peptide inhibitor, D-JNKI-1, can provide neuroprotection against NMDA-induced neurite atrophy and synapse loss.

4. 2 Study Designs

4.2.1 Effects of NOS1AP/nNOS inhibition on NMDA/glycine-induced reduction in outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and L-TAT peptides (1 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis. The TAT fused peptides used in this study included L-TAT alone, L-TAT-GASA and L-TAT GESV.

The sequences of each of these peptides are as follows – L-TAT: NH₂-GRKKRRQRRR-COOH; L-TAT-GASA: NH₂-GRKKRRQRRRYAGQWGASA-COOH; and L-TAT-GESV: NH₂-GRKKRRQRRRYAGQWGESV-COOH, where L-TAT refers to the sequence from HIV-TAT protein which confers cell membrane translocation properties to the peptide; L-TAT-GASA is a negative control in which the critical C-terminal and C-2 position amino acids are replaced by alanine, and finally, the active peptide L-TAT GESV which is composed of TAT fused to 9 amino acids which bind to the PDZ domain pocket of nNOS. L-TAT peptide concentrations were selected on the basis that L-TAT-GESV was capable of blocking NMDA-induced recruitment of NOS1AP to nNOS and NMDA-induced activation of p38 MAPK at 1 μ M in primary cortical neurons at 8-10 DIV (Li et al., 2013).

4.2.2 Effects of L-TAT-GESV on SNP-induced reduction in outgrowth

Primary cortical neurons (DIV 3) were pre-treated with SNP (100 and 300 μ M) for 1 h followed by treatment with a combination of SNP with L-TAT-GESV (1 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis. Concentrations of SNP were based on previously published reports indicating their effects on neurons *in vitro* over a similar range of concentrations (Dawson et al., 1991) and based on our own observations from chapter 3.

4.2.3 Effects of p38 MAPK inhibition on NMDA/glycine-induced reduction in outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and SB203580 (1 μ M), BIRB796 (1 μ M) or L-TAT-D1 (1 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis. SB203580 is known to inhibit the P38 α and p38 β isoforms while BIRB796 inhibits all p38 MAPK isoforms (Kuma et al., 2005). Concentrations of p38 MAPK inhibitors were based on previous data from the literature indicating that SB203580 (1 μ M) can protect against glutamate-induced pyknosis in cerebellar granule neuron extracts without affecting JNK activity (Cao et al., 2004, Cao et al., 2005). BIRB796 was also assessed at a concentration of 1 μ M based on research showing its effects on excitotoxic mechanisms in cortical neuronal cultures (Li et al., 2013). In addition, we tested the experimental peptide, L-TAT-D1 at 1 μ M for

comparative purposes. The full amino acid sequence of L-TAT-D1 is not disclosed, but its actions are mediated through inhibiting NOS1AP dependent recruitment of the p38 MAPK pathway based on unpublished *in vitro* data (Courtney, pers. comm.)

4.2.4 Effects of JNK inhibition on NMDA/glycine-induced reduction in outgrowth

Primary cortical neurons (DIV 3) were pre-treated with NMDA (250 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (250 μ M)/glycine (10 μ M) and the JNK peptide inhibitor D-JNKI-1 (1 μ M and 3 μ M) for 23 h before fixation and immunocytochemistry for Sholl analysis. This peptide was developed by linking the HIV TAT(48-57) transporter sequence to the 20 amino acid JNK binding motif of JNK-interacting protein-1/islet-brain 1 (JIP-1/IB1) (Bonny et al., 2001, Borsello et al., 2003). The sequence of D-JNKI-1 is as follows: d-(DQSRPVQPFLNLTPRKPRPPRRRQRRKKR)G-NH₂. Concentrations were chosen based on research reporting that D-JNKI-1 is capable of protecting primary cortical neurons at DIV 12 against NMDA (50 μ M) induced lactate dehydrogenase (LDH) release at a concentration range of 1 - 4 μ M.

4.2.5 Effects of NOS1AP/nNOS inhibition, p38 MAPK inhibition and JNK inhibition on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

Mature neuronal cultures were used for the investigation of synapse number based on recommended protocols and previous studies in the literature (Ippolito and Eroglu, 2010, Roqué et al., 2014). Primary cortical neurons (DIV 20) were pre-treated with NMDA (10 μ M)/glycine (10 μ M) for 1 h followed by treatment with a combination of NMDA (10 μ M)/glycine (10 μ M) and the NOS1AP peptides, p38 MAPK inhibitors or JNK inhibitor, D-JNKI-1, at (1 μ M) for 23 h before fixation and immunocytochemistry for analysis of the number of co-localised synaptophysin and PSD-95 puncta.

4. 3 Results

4. 3.1 Effects of NOS1AP/nNOS inhibition on NMDA/glycine-induced reduction in outgrowth

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,119}=28.84$, $P<0.001$) on the number of primary neurites, an effect of NMDA/Gly ($F_{1,118}=49.7$, $P<0.001$) and a NMDA/Gly x L-TAT peptides interaction ($F_{3,119}=3.08$, $P=0.03$) on the neuritic length. A two-way ANOVA also demonstrated an effect of NMDA/Gly ($F_{1,119}=39.62$, $P<0.001$), an effect of L-TAT-GESV ($F_{3,119}=3.7$, $P=0.01$) and a NMDA/Gly x L-TAT peptides interaction ($F_{3,119}=3.9$, $P=0.01$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA-treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with the control peptides, L-TAT and L-TAT GASA were unable to reverse against these deficits. However, a significant increase was observed in neuritic length ($P<0.01$) and branches ($P<0.05$) of neurons treated with NMDA/Gly and L-TAT-GESV when compared to NMDA/Gly-treated neurons alone. A three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of L-TAT-GESV ($F_{1,58}=0.96$, $P<0.001$), NMDA/Gly x L-TAT-GESV ($F_{1,58}=8.98$, $P=0.004$), distance ($F_{19,1102}=611.39$, $P<0.001$), NMDA/Gly x distance ($F_{19,1102}=2.82$, $P<0.001$) and L-TAT-GESV x distance ($F_{19,1102}=9.74$, $P<0.001$) and a NMDA/Gly x L-TAT-GESV x distance interaction ($F_{19,1102}=2.15$, $P<0.003$). *Post hoc* analysis revealed that L-TAT-GESV increased the number of intersections at 10 μm ($P<0.05$). NMDA/Gly decreased the number of branches at 10-140 μm ($P<0.01$) and 150-160 μm ($P<0.05$) when compared to control-treated neurons. Treatment with L-TAT-GESV reversed this decrease at 10-60 μm ($P<0.01$) and 70 μm ($P<0.05$) (**Figure 4.1**).

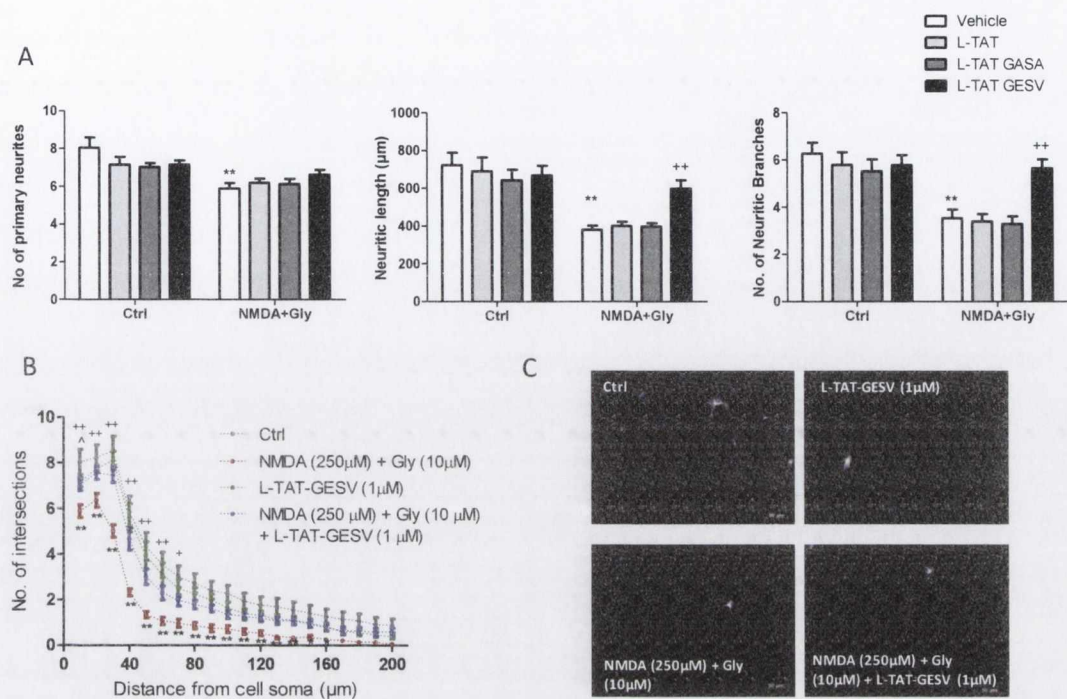


Figure 4.1: Effects of NOS1AP/nNOS inhibition on NMDA/glycine-induced reduction in neurite outgrowth Primary cortical neurons (DIV 3) were treated for 24 h with a combination of NMDA (250 μM)/Gly (10 μM) and L-TAT, L-TAT-GASA or L-TAT-GESV peptides (1 μM). Sholl analysis was performed on neurons to examine **(A)** the number of primary neurites, neuritic length and the number of branches and **(B)** the Sholl profile. Data are expressed as mean ± SEM, n=16 coverslips per group representative of 3 independent experiments. **P<0.01 vs. ctrl, ++P<0.01 vs. respective vehicle (Student-Newman-Keuls). Sholl profile, **P<0.01 and *P<0.05 NMDA 250/Gly vs. ctrl, ++P<0.01 and +P<0.05 NMDA 250/Gly/L-TAT-GESV vs. NMDA 250/Gly, ^P<0.05 L-TAT-GESV vs. ctrl (Student-Newman-Keuls). **(C)** Representative images of neurons identified using the neuronal structural protein β-III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μm.

4.3.2 Effects of L-TAT-GESV on SNP-induced reduction in outgrowth

Two-way ANOVA demonstrated an effect of SNP on the number of primary neurites ($F_{2,90}=18.77$, $P<0.001$), the neuritic length ($F_{2,90}=69.73$, $P<0.001$) and the neuritic branches ($F_{2,90}=52.9$, $P<0.001$), but failed to demonstrate an effect of L-TAT-GESV or a SNP x L-TAT-GESV interaction. *Post hoc* analysis revealed that SNP-treated neurons had fewer primary neurites ($P<0.05$), decreased neuritic length and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons and treatment with L-TAT-GESV failed to reverse these changes (Figure 4.2).

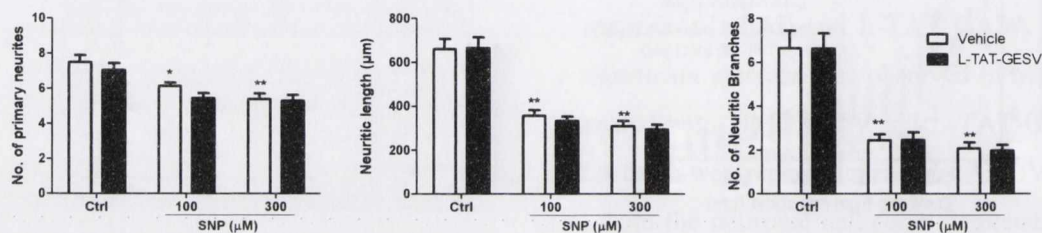


Figure 4.2: Effects of L-TAT-GESV on SNP-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of SNP (100 or 300 μM) and L-TAT-GESV (1 μM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length and the number of branches. Data are expressed as mean ± SEM, n=18 coverslips per group representative of 3 independent experiments. **P<0.01, *P<0.05 vs. ctrl Vehicle (Student-Newman-Keuls).

4.3.3 Effects of p38 MAPK inhibition on NMDA/glycine-induced reduction in neurite outgrowth

4.3.3.1 Effects of SB203580 on NMDA/glycine-induced reduction in neurite outgrowth

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,89}=44.15$, $P<0.001$), an effect of SB203580 ($F_{1,89}=21.9$, $P<0.001$) and a NMDA/Gly x SB203580 interaction ($F_{1,89}=0.78$, $P=0.03$) on the number of primary neurites. A two-way ANOVA also demonstrated an effect of NMDA/Gly ($F_{1,87}=22.3$, $P<0.001$) and an effect of SB203580 ($F_{1,87}=11.5$, $P=0.001$) on the neuritic length. Finally, a two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,87}=25.71$, $P<0.001$) and an effect of SB203580 ($F_{1,87}=4.07$, $P=0.05$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA-treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with SB203580 was able to reverse these deficits, as evidenced by increased number of primary neurites, neuritic length ($P<0.01$) and branches ($P<0.05$) of neurons treated with NMDA/Gly and SB203580 when compared to NMDA/Gly-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,87}=13.4$, $P=0.004$), SB203580 ($F_{1,87}=28.03$, $P<0.001$), distance ($F_{19,1653}=597.17$, $P<0.01$), NMDA/Gly x distance ($F_{19,1653}=5.87$, $P<0.001$) and SB203580 x distance ($F_{19,1653}=12.3$, $P<0.001$). *Post hoc* analysis revealed that SB203580 increased the number of neuritic branches at 10-80 μm ($P<0.01$) and 90 μm ($P<0.05$) compared to control-treated neurons. In contrast, NMDA/Gly decreased the number of branches at 10-50 μm ($P<0.01$) and 60 μm ($P<0.05$) when compared to control-treated neurons. Treatment with SB203580 reversed this decrease at 10-30 μm ($P<0.01$) and 40 μm ($P<0.05$) (**Figure 4.3**).

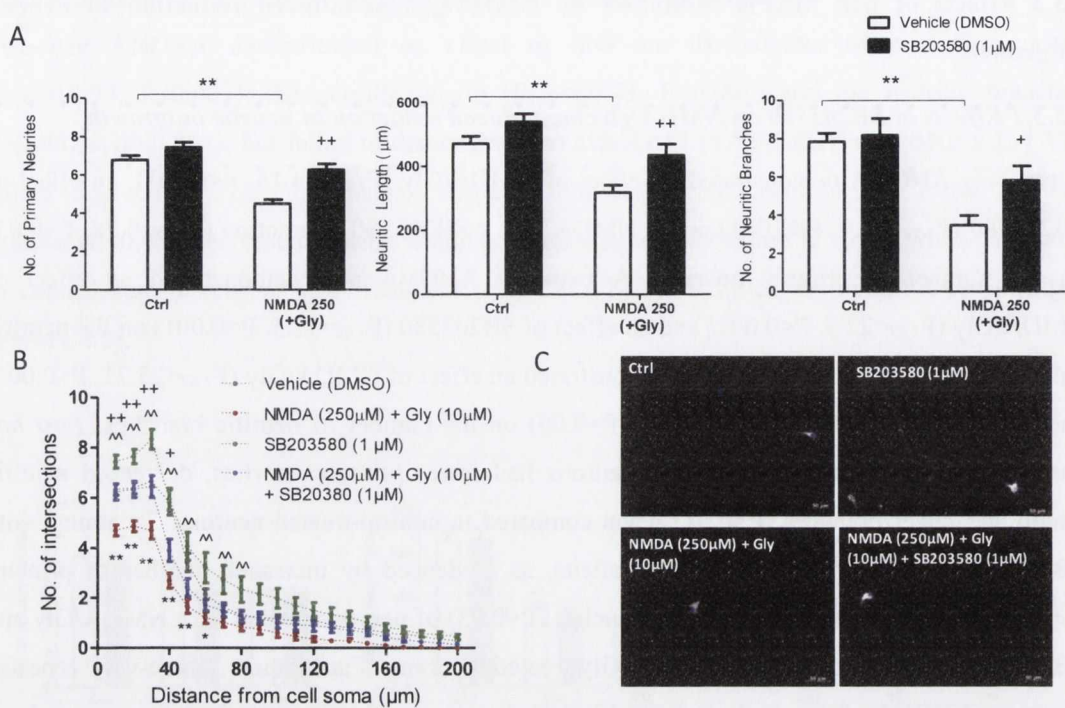


Figure 4.3: Effects of SB203580 on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of NMDA (250 μ M)/Gly (10 μ M) and SB203580 (1 μ M). Sholl analysis was performed on neurons to examine **(A)** the number of primary neurites, neuritic length, number of branches and **(B)** the Sholl profile. Data are expressed as mean $\pm$ SEM, $n=21$ -24 coverslips per group representative of 3 independent experiments. $**P<0.01$ vs. ctrl, $^{++}P<0.01$, $^{+}P<0.05$ vs. respective vehicle (Student-Newman-Keuls). Sholl profile, $**P<0.01$ and $*P<0.05$ NMDA 250/Gly vs. ctrl, $^{++}P<0.01$ and $^{+}P<0.05$ NMDA 250/Gly/SB203580 vs. NMDA 250/Gly, $^{\wedge}P<0.01$ and $^{\wedge}P<0.05$ SB203580 vs. ctrl (Student-Newman-Keuls). **(C)** Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μ m.

4.3.3.2 Effects of BIRB796 on NMDA/glycine-induced reduction in neurite outgrowth

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,91}=26.99$, $P<0.001$), an effect of BIRB796 ($F_{1,91}=31.95$, $P<0.001$) and a NMDA/Gly x BIRB796 interaction ($F_{1,91}=5.27$, $P=0.02$) on the number of primary neurites. A two-way ANOVA also demonstrated an effect of NMDA/Gly ($F_{1,90}=27.38$, $P<0.001$), an effect of BIRB796 ($F_{1,90}=42.43$, $P<0.001$) and a NMDA/Gly x BIRB796 interaction ($F_{1,90}=11.535$, $P=0.001$) on the neuritic length. In addition, a two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,89}=22.7$, $P<0.001$) and an effect of BIRB796 ($F_{1,87}=7.4$, $P=0.008$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA-treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with BIRB796 was able to reverse these deficits, as shown by increased number of primary neurites, neuritic length ($P<0.01$) and branches ($P<0.05$) of neurons treated with NMDA/Gly and BIRB796 when compared to NMDA/Gly-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,90}=35.77$, $P<0.001$), BIRB796 ($F_{1,90}=28.28$, $P<0.001$), distance ($F_{19,1710}=548.34$, $P<0.01$), NMDA/Gly x distance ($F_{19,1710}=10.83$, $P<0.001$) and BIRB796 x distance ($F_{19,1710}=11.45$, $P<0.001$). *Post hoc* analysis revealed that BIRB796 increased the number of neuritic branches at 10 μm and 30-80 μm ($P<0.01$) as well as at 20 μm and 90 μm ($P<0.05$) compared to untreated control neurons. In contrast, NMDA/Gly decreased the number of branches at 10-50 μm ($P<0.01$) and 60 μm ($P<0.05$) when compared to control-treated neurons. Treatment with BIRB796 reversed this decrease at 10-60 μm and 90 μm ($P<0.01$) and also at 70 μm , 80 μm and 100 μm ($P<0.05$) (**Figure 4.4.**).

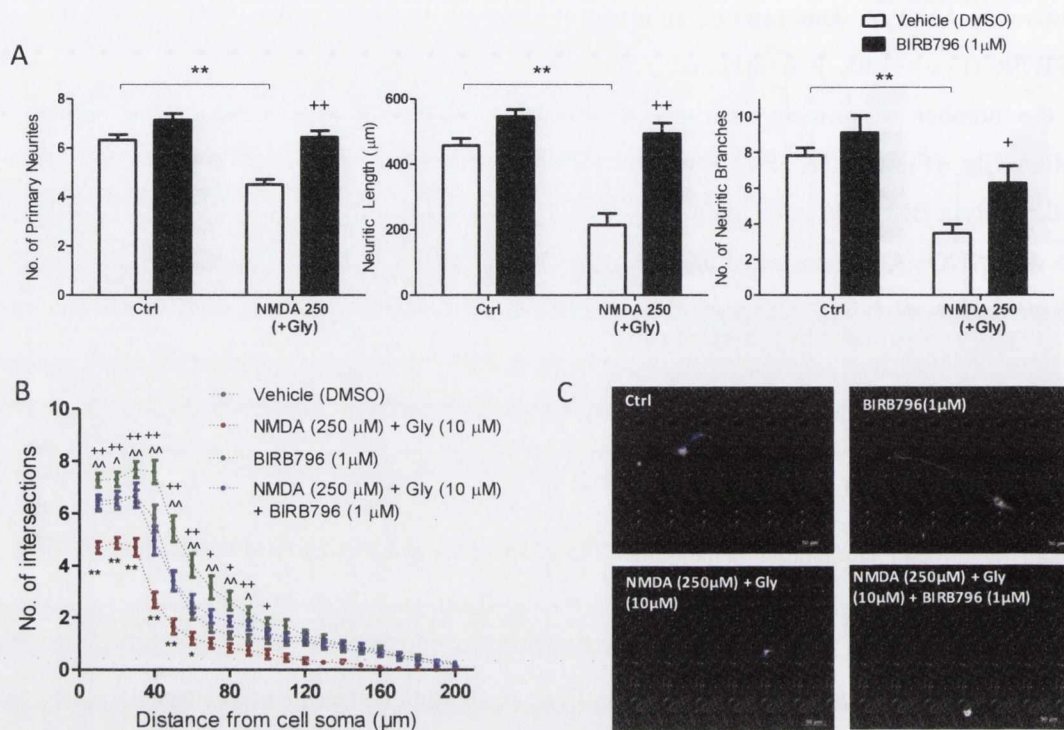


Figure 4.4: Effects of BIRB796 on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of NMDA (250 μ M)/Gly (10 μ M) and BIRB796 (1 μ M). Sholl analysis was performed on neurons to examine **(A)** the number of primary neurites, neuritic length, number of branches and **(B)** the Sholl profile. Data are expressed as mean $\pm$ SEM, $n=23-24$ coverslips per group representative of 3 independent experiments. $**P<0.01$ vs. ctrl, $^{++}P<0.01$, $^{+}P<0.05$ vs. respective vehicle (Student-Newman-Keuls). Sholl profile, $**P<0.01$ and $^{*}P<0.05$ NMDA 250/Gly vs. ctrl, $^{++}P<0.01$ and $^{+}P<0.05$ NMDA 250/Gly/BIRB796 vs. NMDA 250/Gly, $^{^^}P<0.01$ and $^{^}P<0.05$ BIRB796 vs. ctrl (Student-Newman-Keuls). **(C)** Representative images of neurons identified using the neuronal structural protein β -III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μ m.

4.3.3.3 Effects of L-TAT-D1 on NMDA/glycine-induced reduction in neurite outgrowth

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,125}=23.4$, $P<0.001$), an effect of TAT peptides ($F_{2,125}=7.69$, $P<0.001$) and a NMDA/Gly x TAT peptide interaction ($F_{2,125}=5.45$, $P=0.005$) on the number of primary neurites. A two-way ANOVA also demonstrated an effect of NMDA/Gly ($F_{1,125}=30.17$, $P<0.001$), an effect of TAT peptides ($F_{2,125}=6.04$, $P=0.003$) and a NMDA/Gly x TAT peptides interaction ($F_{2,125}=14.56$, $P<0.001$) on the neuritic length. Similarly, a two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,125}=24.52$, $P<0.001$) and an effect of TAT peptides ($F_{2,125}=11.57$, $P<0.001$), as well as a NMDA/Gly x TAT peptides interaction ($F_{2,125}=9.68$, $P=0.001$) on the number of neuritic branches. *Post hoc* analysis revealed that NMDA-treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with L-TAT failed to reverse this deficit, as seen by a decrease in primary neurites, neuritic length and number of branches compared to control treated neurons. Treatment with L-TAT-D1 however was able to reverse these deficits, as evidenced by increased number of primary neurites, neuritic length ($P<0.01$) and branches ($P<0.05$) of neurons treated with NMDA/Gly and BIRB796 when compared to NMDA/Gly-treated neurons. Furthermore, three-way repeated measures ANOVA of the number of neuritic branches at specific distances from the neuronal cell soma demonstrated effects of NMDA/Gly ($F_{1,82}=7.64$, $P=0.007$), L-TAT-D1 ($F_{1,82}=15.36$, $P<0.001$), NMDA/Gly x L-TAT-D1 ($F_{1,82}=18.35$, $P<0.001$), distance ($F_{19,1558}=458.39$, $P<0.001$), NMDA/Gly x distance ($F_{19,1558}=7.24$, $P<0.001$) and L-TAT-D1 x distance ($F_{19,1558}=7.09$, $P<0.001$) and a NMDA/Gly x TAT-D1 x distance interaction ($F_{19,1558}=4.25$, $P<0.001$). *Post hoc* analysis revealed that L-TAT-D1 increased the number of neuritic branches at 10-50 μm ($P<0.01$) and 60 μm ($P<0.05$) compared to untreated control neurons. In contrast, NMDA/Gly decreased the number of branches at 10-80 μm ($P<0.01$) when compared to control-treated neurons. Treatment with L-TAT-D1 reversed this decrease at 10-60 μm ($P<0.01$) (**Figure 4.5**).

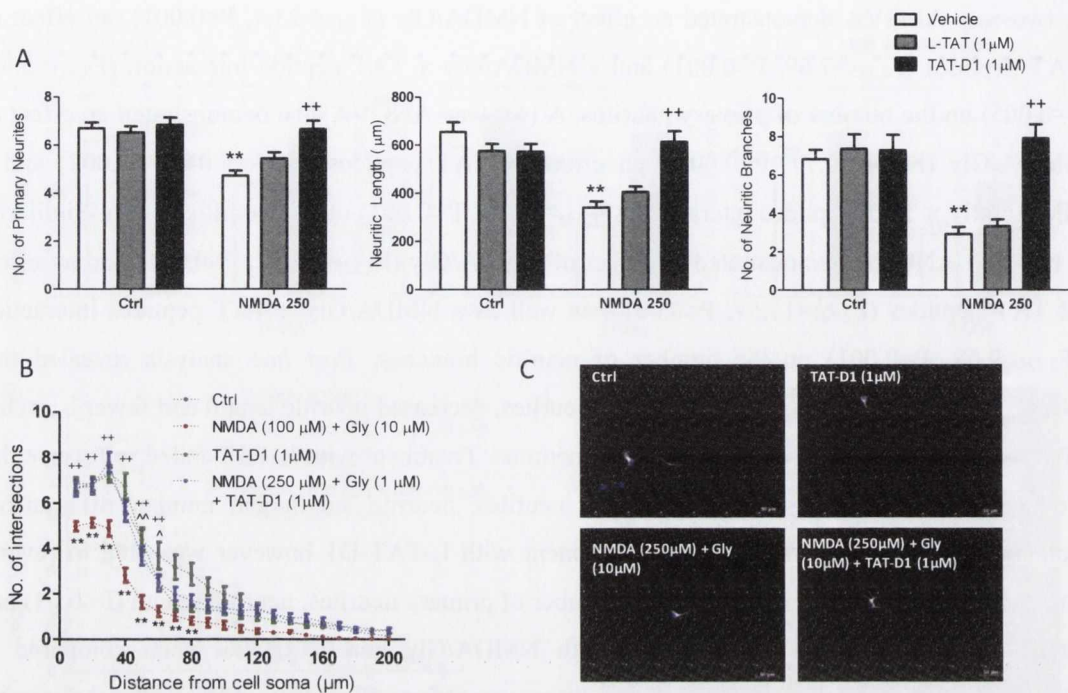


Figure 4.5: Effects of L-TAT-D1 on NMDA/glycine-induced reduction in neurite outgrowth Primary cortical neurons (DIV 3) were treated for 24 h with a combination of NMDA (250 μM)/Gly (10 μM), L-TAT (1 μM) or TAT-D1 (1 μM). Sholl analysis was performed on neurons to examine (A) the number of primary neurites, neuritic length, number of branches and (B) the Sholl profile. Data are expressed as mean ± SEM, n=20-24 coverslips per group representative of 3 independent experiments. **P<0.01 vs. ctrl, ++P<0.01 vs. respective vehicle (Student-Newman-Keuls). Sholl profile, **P<0.01 NMDA 250/Gly vs. ctrl, ++P<0.01 and NMDA 250/Gly/TAT-D1 vs. NMDA 250/Gly, ^P<0.01 and ^P<0.05 TAT-D1 vs. ctrl (Student-Newman-Keuls). (C) Representative images of neurons identified using the neuronal structural protein β-III tubulin (white) and the DAPI nuclear stain (blue) with fluorescence microscopy, Scale bar = 50 μm.

4.3.4 Effects of D-JNKI-1 on NMDA/glycine-induced reduction in neurite outgrowth

Two-way ANOVA demonstrated an effect of NMDA/Gly on the number of primary neurites ($F_{1,102}=25.96$, $P<0.001$), the neuritic length ($F_{1,102}=35.42$, $P<0.001$) and the neuritic branches ($F_{1,102}=55.2$, $P<0.001$), but failed to demonstrate an effect of peptide or NMDA/Gly x peptide interaction. *Post hoc* analysis revealed that NMDA/Gly -treated neurons had fewer primary neurites, decreased neuritic length and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons and treatment with the L-TAT control peptide or the D-JNKI-1 peptide failed to reverse these deficits (**Figure 4.6A**).

D-JNKI-1 at the higher concentration of 3 μ M also failed to reverse NMDA/Gly induced deficits in neurite outgrowth. Two-way ANOVA demonstrated an effect of NMDA/Gly on the number of primary neurites ($F_{1,102}=35.15$, $P<0.001$), the neuritic length ($F_{1,101}=56.6$, $P<0.001$) and the neuritic branches ($F_{1,102}=29.23$, $P<0.001$), but failed to demonstrate an effect of peptide or NMDA/Gly x peptide interaction. *Post hoc* analysis revealed that NMDA/Gly -treated neurons had fewer primary neurites, decreased neuritic length and fewer neuritic branches ($P<0.01$) when compared to control-treated neurons and treatment with the L-TAT control peptide or the D-JNKI-1 peptide failed to reverse these deficits (**Figure 4.6B**).

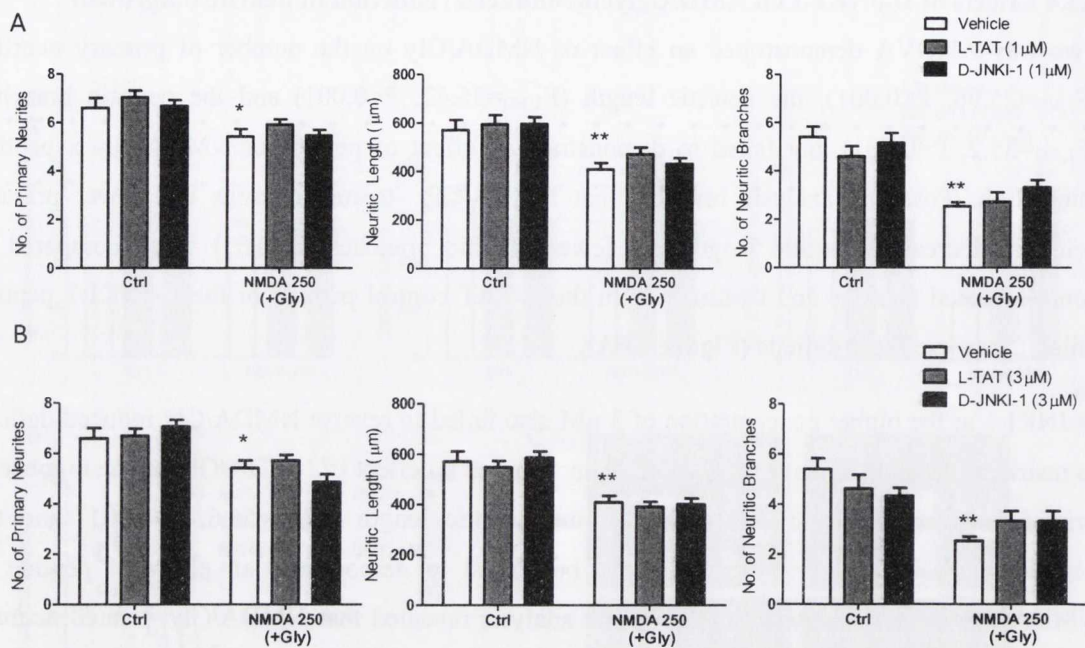


Figure 4.6: Effects of D-JNKI-1 on NMDA/glycine-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of NMDA (250 μM) /Gly (1 μM) and **(A)** L-TAT (1 μM) and D-JNKI-1 (1 μM) or **(B)** L-TAT (3 μM) and D-JNKI-1 (3 μM). Sholl analysis was performed on neurons to examine the number of primary neurites, neuritic length and the number of branches. Data are expressed as mean ± SEM, n=18 coverslips per group representative of 3 independent experiments. **P<0.01, *P<0.05 vs. respective ctrl (Student-Newman-Keuls).

4.3.5 Effects of NOS1AP/nNOS inhibition, p38MAPK inhibition and JNK inhibition on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

Since L-TAT-GESV and p38 MAPK inhibition significantly attenuated NMDA-induced deficits in neurite outgrowth in immature (DIV 4) cortical neurons, the effects of these compounds on the co-localised expression of pre-synaptic synaptophysin and post-synaptic PSD-95 in mature neuronal cultures (DIV 20) was investigated as a measure of synapse number (**Figure 4.7A**).

4.3.5.1 Effects of NOS1AP/nNOS inhibition on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,356}=34.5$, $P<0.001$), an effect of L-TAT peptides ($F_{3,356}=5.97$, $P<0.001$) and a NMDA/Gly x L-TAT peptide interaction ($F_{3,356}=4.02$, $P=0.007$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised puncta than control neurons. Co-treatment with L-TAT and L-TAT-GASA failed to reverse these NMDA/Gly-induced deficits. However, *post hoc* analysis revealed that treatment with L-TAT-GESV attenuated NMDA/Gly-induced decreases as evidenced by an increase in co-localised puncta ($P<0.01$) in neurons treated with NMDA/Gly and L-TAT-GESV when compared to NMDA/Gly-treated neurons alone ($P<0.01$) (**Figure 4.7B**).

4.3.5.2 Effects of SB203580 on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,182}=7.84$, $P=0.006$), an effect of SB203580 ($F_{1,182}=12.02$, $P<0.001$) and a NMDA/Gly x SB203580 interaction ($F_{1,182}=6.59$, $P=0.01$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised puncta when compared to control treated neurons ($P<0.01$). Treatment with SB203580 attenuated this reduction as evidenced by a significant increase in co-localised puncta ($P<0.01$) in neurons treated with NMDA/Gly and SB203580 when compared to NMDA/Gly-treated neurons alone ($P<0.01$) (**Figure 4.7C**).

4.3.5.3 Effects of BIRB796 on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,192}=16.97$, $P<0.001$), an effect of BIRB796 ($F_{1,192}=10.07$, $P=0.002$) and a NMDA/Gly x BIRB796 interaction ($F_{1,192}=11.99$, $P<0.001$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised puncta than control neurons. Co-treatment with BIRB796 attenuated NMDA/Gly-induced decreases as evidenced by an increase in co-localised puncta ($P<0.01$) in neurons treated with NMDA/Gly and BIRB796 when compared to NMDA/Gly-treated neurons alone ($P<0.01$) (**Figure 4.7D**).

4.3.5.4 Effects of L-TAT-D1 on NMDA/glycine-induced reduction in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,278}=22.17$, $P<0.001$), and a NMDA/Gly x L-TAT peptides interaction ($F_{2,278}=8.28$, $P<0.001$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised puncta when compared to control treated neurons ($P<0.01$). Co-treatment with L-TAT failed to reverse these NMDA/Gly-induced deficits. In contrast, treatment with L-TAT-D1 attenuated this decrease as evidenced by an increase in the number of co-localised puncta in neurons treated with NMDA/Gly + TAT-D1 when compared to NMDA/Gly treated neurons alone ($P<0.05$) (**Figure 4.7E**).

4.3.5.5 Effects of D-JNKI-1 on NMDA/glycine-induced reductions in the co-localised expression of synaptophysin and PSD-95

A two-way ANOVA demonstrated an effect of NMDA/Gly ($F_{1,291}=29.06$, $P<0.001$), an effect of TAT peptides ($F_{2,291}=8.19$, $P<0.001$) and a NMDA/Gly x TAT peptides interaction ($F_{2,291}=8.62$, $P<0.001$) on the number of co-localised synaptophysin and PSD-95 puncta. *Post hoc* analysis revealed that NMDA/Gly-treated neurons had fewer co-localised puncta when compared to control treated neurons ($P<0.01$). Again, co-treatment with L-TAT failed to reverse NMDA/Gly-induced deficits. However, treatment with D-JNKI-1 attenuated this decrease as evidenced by an increase in the number of co-localised puncta in neurons treated with NMDA/Gly + TAT-D1 when compared to NMDA/Gly treated neurons alone ($P<0.01$) (**Figure 4.7F**).

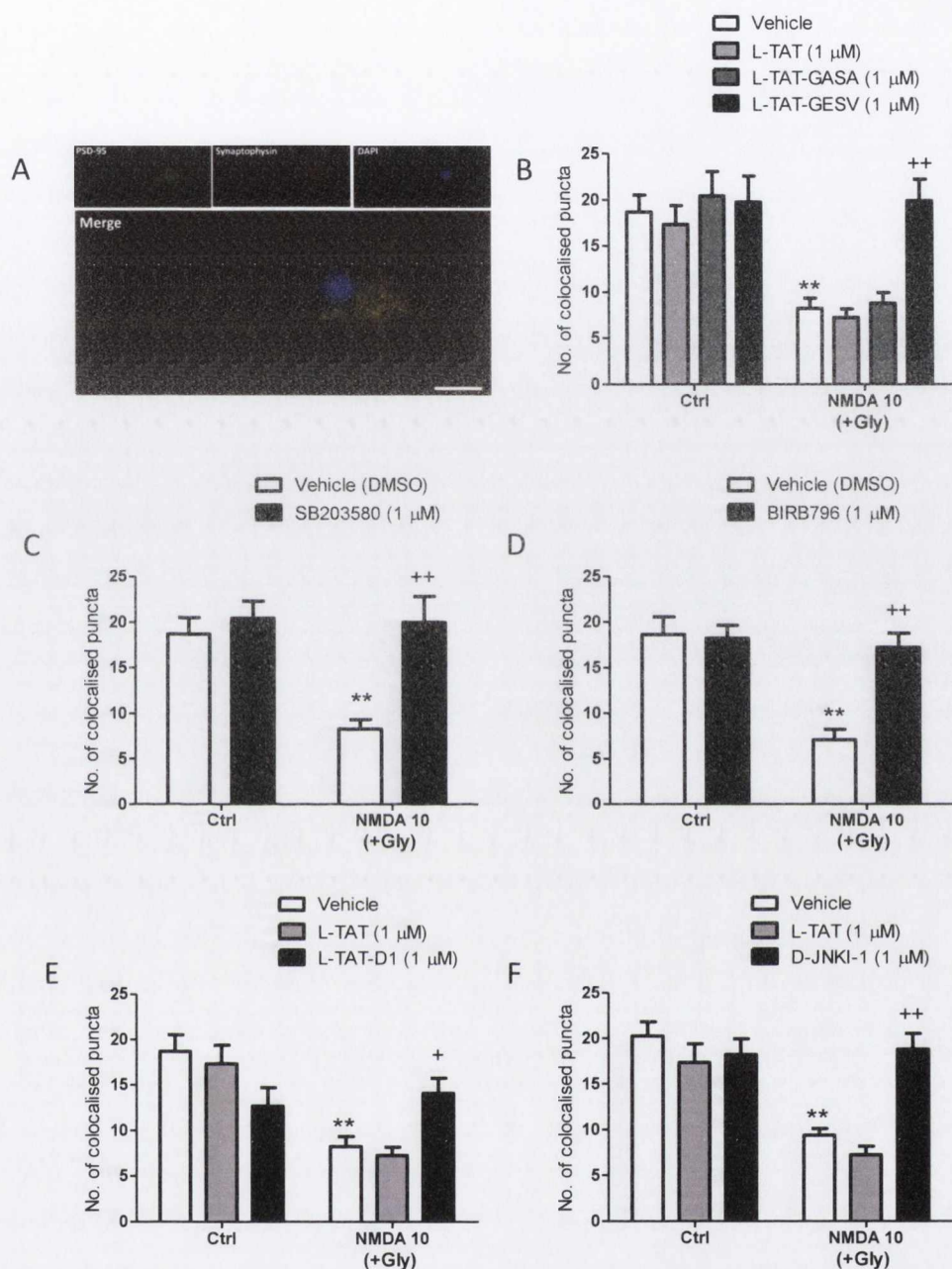


Figure 4.7: Effects of NOS1AP/nNOS inhibition, p38 MAPK inhibition, and JNK inhibition on NMDA/glycine-induced reductions in the co-localised expression of synaptophysin and PSD-95. (A) Representative image of control-treated primary cortical neurons (DIV 21) identified using the post-synaptic marker PSD-95 (green), the pre-synaptic marker synaptophysin (red) and the DAPI nuclear stain (blue) with fluorescence microscopy. Quantification of co-localised puncta was conducted using ImageJ software. Scale Bar = 20 μ m. Neurons (DIV 20) were treated for 24 h with a combination of NMDA (10 μ M)/Gly (10 μ M) and (B) L-TAT-GESV (1 μ M), (C) SB203580 (1 μ M), (D) BIRB796 (1 μ M), (E) L-TAT-D1 (1 μ M), or (F) D-JNKI-1 (1 μ M). Data are expressed as mean $\pm$ SEM, n=42-52 neurons per group representative of 3 independent experiments. **P<0.01 vs. ctrl, (Student-Newman-Keuls).

4.4 Discussion

The results of the current investigation demonstrated that NOS1AP plays a role in neurite outgrowth and that inhibition of the NOS1AP/nNOS interaction through the use of a TAT-fused CPP, L-TAT-GESV, protected against both NMDA-induced reduction in neurite outgrowth and synaptic protein co-localisation, which is taken as a measure of synapse number. Furthermore, we demonstrated a preferential involvement of p38 MAPK activation in NMDA/glycine-induced neurite atrophy by showing that p38 MAPK inhibition, but not JNK inhibition, protected against NMDA-induced effects. In this regard we showed that the commercial p38 MAPK inhibitors, SB203580 and BIRB796, as well as an experimental CPP inhibitor of the p38 MAPK pathway, TAT-D1, prevented NMDA-induced neuronal atrophy in primary neuronal cultures, whereas JNK inhibition by the CPP, D-JNNKI-1, cannot protect against these effects. In contrast, we demonstrated that both p38 MAPK inhibition and JNK inhibition attenuated NMDA-induced reduction in the co-localised expression of synaptic proteins, suggesting different mechanisms underlying the processes of NMDA-induced neurite atrophy and NMDA-induced potential synapse loss.

4.4.1 L-TAT-GESV protects against NMDA-induced neuronal atrophy

The results of this investigation demonstrated that disruption of the NOS1AP/nNOS interaction using a cell permeable peptide inhibitor, L-TAT-GESV, significantly attenuated NMDA-induced neuronal atrophy in primary cortical neurons (DIV 3). More specifically, neurons treated with NMDA (250 μ M) and glycine (10 μ M) in addition to L-TAT-GESV (1 μ M) showed significant increases in neuritic length and displayed significantly more neuritic branches at 10-70 μ m from the cell soma compared to NMDA/glycine-treated neurons alone. Neurons were also treated with control peptides consisting firstly of the transduction sequence alone (L-TAT) and secondly with a mutant peptide lacking the sequence specificity needed for binding to nNOS (L-TAT-GASA). In contrast to the results observed with L-TAT-GESV, the treatment of neurons with L-TAT and L-TAT-GASA failed to offer the same protection against NMDA/glycine in terms of increases in neuritic length and neuritic branches. These results demonstrate that the -GESV binding motif is required for the observed neuroprotective effects and helps to confirm that L-TAT-GESV is acting specifically by blocking the NOS1AP/nNOS interaction to produce neuroprotective effects rather than potential off-target effects associated with TAT-mediated peptide delivery. These results indicate that the NOS1AP/nNOS protein interaction acts to mediate NMDA signalling, and shows that the inhibition of NOS1AP/nNOS can prevent detrimental effects associated with excess NMDA. Such results are consistent with recent reports which demonstrated the efficacy of uncoupling NOS1AP from nNOS using L-TAT-GESV *in vitro* (Li et al., 2013). These authors used primary neuronal cultures at DIV 8-10 and found that L-TAT-GESV (1 μ M) significantly inhibited the recruitment of NOS1AP to nNOS and protected against NMDA-evoked toxicity as

measured by both propidium iodide (PI) staining which measures damage to the soma, in addition to the more sensitive LDH release assay which measures cytoplasmic leakage.

L-TAT-GESV has also shown promising effects *in vivo* in an animal model of neonatal cerebral hypoxia-ischaemia (Li et al., 2013). For these *in vivo* studies, the D-form inverse amino acid sequence of TAT (D-TAT) was used instead of L form TAT (L-TAT), as the D form confers higher stability properties to the peptide and reduces breakdown by proteases. In this model, D-TAT-GESV (100 ng) was shown to be capable of entering neurons in both striatum and cortex 6 h following intracerebroventricular injection. The peptide was capable of attenuating increases in phospho p38 activation occurring 30 min after hypoxia-ischaemia and provided long lasting neuroprotection which lasted one week following the ischaemic insult as observed by an increase in surviving tissue. These neuroprotective effects were comparable to those observed using SB203580, suggesting that targeting the NOS1AP/nNOS interaction provides considerable neuroprotection and may offer a novel therapeutic target for the treatment of ischaemia.

To further investigate the specificity of L-TAT-GESV we investigated downstream of the NOS1AP/nNOS protein interaction and examined whether L-TAT-GESV could rescue against reductions in outgrowth associated with excessive production of NO induced by SNP (100 and 300 μ M). We hypothesised that L-TAT-GESV would be ineffective in this case, since SNP does not depend on upstream NMDA-evoked effects to produce excess NO, and hence bypasses the requirement of NOS1AP/nNOS related signalling. The results demonstrated that L-TAT-GESV fails to rescue against SNP-induced decreases in the number of primary neurites, neuritic length or number of neuritic branches. These results further indicate that L-TAT-GESV acts specifically to inhibit the nNOS and NOS1AP protein interaction, and selectively inhibits NMDA-evoked effects, rather than inhibiting downstream nNOS catalytic activity or NO mediated cellular responses. These results are consistent with previous results using this peptide (Li et al., 2013), and are also consistent with our own research from the previous chapter which showed that small-molecule inhibition of the PSD-95/nNOS interaction also failed to protect against SNP-mediated decreases in neurite outgrowth.

In addition to neurite outgrowth, we also investigated the potential of this peptide to protect against reductions in synaptic protein co-localisations induced by NMDA in mature neurons (DIV 20). Synapse loss is a prominent pathological feature of many neurological disorders associated with altered glutamatergic signalling such as Alzheimer's disease (Shankar et al., 2007) and major depression (Tata and Anderson, 2010, Duman and Aghajanian, 2012). The results of this investigation showed that L-TAT-GESV (1 μ M) significantly attenuated the reduction in synaptic protein co-localisation observed following treatment with NMDA (10 μ M) and glycine (10 μ M). These results may be of interest for research into illnesses associated with synaptic deficits. Most

notably, compelling evidence suggests that the NMDA-R antagonist ketamine may elicit its well known antidepressant effects through increasing synaptogenesis (Li et al., 2010, Li et al., 2011). It is therefore of interest that the inhibition of pathways downstream of NMDA-R, such as inhibition of the NOS1AP/nNOS interaction, can potentially increase synapse number in an *in vitro* model. These results suggest that the NOS1AP/nNOS pathway may be a relevant target for protecting against synaptic deficits reported in such disorders.

4.4.2 p38 MAPK inhibitors protect against NMDA-induced neuronal atrophy

We were interested in investigating whether inhibition of the SAPKs, p38 MAPK and/or JNK, could protect against the observed reductions in outgrowth and synapse number induced by excess NMDA. The results of the present investigation demonstrated that both the p38 MAPK inhibitors SB203580 and BIRB796 (1 μ M) prevented NMDA/glycine-induced neurite regression by increasing the number of primary neurites and the neuritic length compared to NMDA/glycine treated neurons. Furthermore, SB203580 was capable of attenuating against the NMDA/glycine-induced decrease in number of neuritic branching intersections at 10 - 40 μ m from the cell soma, while BIRB796 protected against these deficits at 10 - 90 μ m from the cell soma. We also investigated the effects of these inhibitors in protecting against NMDA/glycine-evoked reductions in synaptic protein co-localisations in mature neuronal cultures (DIV 20), and found that both SB203580 and BIRB796 (1 μ M) significantly protected against reductions in synapse number as measured by an increase in the staining of co-localised synaptophysin and PSD-95 puncta compared to NMDA (10 μ M) and glycine (10 μ M) treated neurons. Such results provide further evidence for a role of p38 MAPK activation in NMDA-induced cell atrophy. These results are in line with previously published reports linking NMDA and NO to the activation of p38 MAPK (Molz et al., 2008, Yang et al., 2012). Previous research has shown that nNOS activation and NO production are critical components of glutamate-induced p38 MAPK activation and that inhibition of nNOS using L-NAME and 7-NI impairs p38 MAPK activation by NMDA in cortical neurons (Cao et al., 2005, Soriano et al., 2008). Importantly, NOS1AP has been shown to recruit the p38 MAPK activating kinase MKK3, and L-TAT-GESV was capable of inhibiting p38 MAPK activation and the subsequent recruitment of cell death pathways (Li et al., 2013).

Considering that SB203580 and BIRB796 were capable of attenuating NMDA/glycine-induced reductions in outgrowth and synaptic protein co-localisation, we investigated the potential of an experimental P38 MAPK peptide inhibitor to produce similar neuroprotective effects. This inhibitor, termed L-TAT-D1, is an L-TAT fused peptide inhibitor which acts by preventing NOS1AP dependent activation of the p38 MAPK pathway. As such, the use of this peptide is thought to provide a more specific means to selectively inhibit NOS1AP/p38 MAPK signalling compared to either SB203580 or BIRB796. The results presented here demonstrated that L-TAT-D1 is as efficacious as SB203580 and BIRB796 in providing protection against NMDA/glycine-

induced atrophic effects. Specifically, we show that L-TAT-D1 (1 μ M) protects immature neurons (DIV 3) against reductions in all parameters of neurite outgrowth by showing an increase in the number of primary neurites, increased neuritic length and an increase in the number of branching points at 10 – 50 μ m from the cell soma compared to NMDA/glycine-treated neurons. Additionally, the results demonstrated that L-TAT-D1 protected against reductions in the co-localised expression of synaptic proteins induced by the treatment of mature neuronal cultures (DIV 20) with NMDA (10 μ M) and glycine (10 μ M). Together, these results suggest that inhibition of p38 MAPK activation provides protection against the detrimental effects associated with excess NMDA on neuronal morphology.

4.4.3 Distinct roles for JNK and p38 MAPKs in NMDA-induced neurite atrophy

We also investigated the involvement of JNK in neurite outgrowth responses and synaptic protein co-localisation by employing the use of the cell permeable peptide, D-JNKI-1. We found that D-JNKI-1 at concentration of 1 μ M and 3 μ M failed to protect against NMDA (250 μ M) and glycine (10 μ M)-induced reductions in outgrowth at DIV 3 when applied 1 h following NMDA/glycine. However, in contrast to the lack of effects observed with D-JNKI-1 in terms of neurite outgrowth in immature neurons, we demonstrated that D-JNKI-1 can rescue against NMDA/glycine-induced reductions in synaptic protein co-localisation in mature neurons at DIV 20. Previous data has reported that D-JNKI-1 is capable of protecting primary cortical neurons at DIV 12 against NMDA (100 μ M) at a concentration range of 2 - 4 μ M and against a milder NMDA stimulus (50 μ M) at a concentration range of 1 - 4 μ M as assessed by LDH release (Borsello et al., 2003). Given the protective role of D-JNKI-1 documented in these studies, it may be of some surprise that D-JNKI-1 failed to show protective effects in our outgrowth model in terms of protection against NMDA (250 μ M) and glycine (10 μ M)-induced neurite atrophy at DIV 3-4. In contrast, our results demonstrated that inhibition of p38 MAPK by two widely used p38 MAPK inhibitors, SB203580 and BIRB796, as well as an experimental p38 MAPK inhibiting peptide, L-TAT-D1, were capable of attenuating NMDA-induced atrophic effects. These results suggest that p38 and JNK pathways may play distinct roles in NMDA-induced neurite outgrowth responses. In this regard, although p38 MAPK and JNK are both stress activated protein kinases that are generally considered to share similar roles in excitotoxic pathways, it has been reported that these two kinases show distinct roles in glutamate-induced neuronal death. Of particular importance, previous research using cerebellar granule cells at DIV 7-9 reported that glutamate-induced apoptosis is accompanied by strong activation of p38 which can be prevented by p38 inhibitors and occurs in a caspase-independent fashion. In contrast, the same study reports that activation of JNK was predominant during caspase-dependent apoptosis which occurs following trophic withdrawal. Interestingly, p38 inhibition failed to protect against trophic withdrawal-induced apoptosis in this model, while JNK inhibition failed to protect against glutamate-induced

apoptosis (Cao et al., 2004). In addition, research working on rat primary cortical neurons found that p38 MAPK inhibition with SB203580 was capable of protecting neurons against NMDA-induced caspase-dependent apoptosis while JNK or ERK inhibition failed to provide protection (Yang et al., 2012). These results suggest different JNK and p38 MAPK activation patterns depending on the stimulus used to induce a stress response (i.e. glutamate induced excitotoxicity versus trophic withdrawal). The results of the present investigation are in line with these reports which indicate preferential p38 MAPK activation following glutamate-induced toxicity and may explain the limited ability of JNK inhibition by D-JNKI-1 to protect against NMDA-induced atrophy.

4.4.4 Validity of NOS1AP/nNOS disruption as a method to provide neuroprotection

Taken together, the results of the present study indicate that the NOS1AP/nNOS interaction plays a role in neurite outgrowth and synaptic morphology in primary cortical neurons *in vitro*. The current investigation employed the use of CPPs as a method to selectively inhibit various proteins along the NOS1AP/nNOS/p38 MAPK pathway. Of particular importance, we demonstrated that a CPP referred to as L-TAT-GESV provided protection against NMDA/glycine-induced decreases in both neurite outgrowth and synaptic protein co-localisation. L-TAT-GESV acts by competing with NOS1AP for binding to nNOS, and is composed of a sequence of 9 amino acids that are based on the PDZ motif of NOS1AP. It is of importance to highlight that a separate region of NOS1AP - the so-called ExF motif - was recently reported to bind to nNOS with far greater affinity than that of the PDZ motif (Li et al., 2015). Supporting this finding, a peptide construct based on the ExF region was found to be as efficacious as L-TAT-GESV in the regulation of excitotoxic pathways. This research suggested that ExF is the primary site of NOS1AP/nNOS binding, while the PDZ motif acts as an important stabilizer for the interaction. These findings highlight that other relevant protein interaction sites exist on NOS1AP which are of significance to NOS1AP/nNOS signalling and suggests multiple discrete sites for potential pharmacological intervention.

Our work also establishes the involvement of p38 MAPK activation in neurite outgrowth and synaptic morphology, and we demonstrated the efficacy of an experimental TAT-fused peptide which acts to prevent NOS1AP-dependent p38 activation in protecting against NMDA-induced neuronal atrophy. Our work suggests a preferential activation of the p38 MAPK branch of stress activated protein kinases in NMDA-induced neurite regression, as p38 inhibition, but not JNK inhibition, was capable of attenuating NMDA/glycine-induced effects on neurite outgrowth. In contrast however, we demonstrated comparable efficacy of both p38 and JNK MAPKs in protecting against NMDA/glycine-induced reductions in synaptic protein co-localisation, suggesting that both of these kinase pathways may be involved in NMDA-related synapse loss in more mature neuronal cultures.

Previous reports have demonstrated that L-TAT-GESV and a similar peptide based on the ExF region of NOS1AP can protect against NMDA-induced excitotoxic cell death (Li et al., 2013, Li et al., 2015). Here, we show for the first time that L-TAT-GESV can also produce neurotrophic effects as measured by increases in neurite outgrowth and complexity in addition to an attenuation of NMDA-induced synapse loss *in vitro*. These neurotrophic effects lend support to the possibility that this peptide may be advantageous in animal models of diseases associated with neurite loss such as neurodegenerative disorders and neuropsychiatric disorders. In addition, this work establishes the role of NOS1AP as a mediator rather than an inhibitor of the PSD-95/nNOS interaction and identifies NOS1AP as an additional downstream site for the production of neuroprotection in addition to the PSD-95/nNOS interaction.

4.4.5 Conclusions

In conclusion, the results of the present investigation demonstrated that inhibiting the NOS1AP/nNOS protein interaction using a CPP construct, which competes with NOS1AP for binding to nNOS, can protect neurons against NMDA/glycine-induced decreases in both neurite outgrowth and in the co-localised expression of synaptic proteins. We demonstrated that NMDA/glycine-induced decreases in neurite outgrowth involve a preferential involvement of the p38 branch of MAPKs, by showing that p38 MAPK inhibitors, SB203580 and BIRB796 can protect against NMDA-induced reductions in neurite outgrowth while JNK inhibition fails to protect against these deficits. We also show that an experimental TAT fused peptide - L-TAT-D1 - which is believed to act by inhibiting the recruitment of p38 to NOS1AP, shows similar efficacy to standard p38 MAPK inhibitors in protecting against the effects of NMDA on neurite outgrowth and synaptic protein co-localisations. This further validates the use of TAT-fused peptides as a means of selectively targeting the NOS1AP/nNOS protein interaction for the production of neuroprotective effects. Our data supports the role of NOS1AP as a mediator of NMDA-R signalling and suggests that targeting the NOS1AP/nNOS protein interaction may offer a promising pharmacological site for the development of new therapeutics for the treatment of CNS disorders associated with excess glutamatergic stimulation and neuronal atrophy.

Chapter 5: Astrocytic ablation by L-alpha aminoadipic acid (L-AAA) induces neuronal atrophy; Rescue by NMDA-R antagonists and inhibitors of the PSD-95/nNOS interaction.

5.1 Introduction

Astrocytes are the most abundant cell type in the CNS and play various roles crucial for the maintenance of normal brain function such as the regulation of brain metabolism and glutamate transmission (Sofroniew and Vinters, 2010). In addition to these homeostatic functions, increasing evidence has shown that astrocytes are important regulators of neuronal growth and dendritic morphology (Matsutani and Yamamoto, 1998, Dijkstra et al., 1999, Johansson and Strömberg, 2002, Previrera et al., 2010). For instance, astrocytes are known to regulate neuronal morphology through the provision of ECM molecules which form a permissive substrate to encourage neuronal guidance and development (Jiang et al., 2007, Previrera et al., 2010). In addition, astrocytes actively secrete soluble factors which enhance neurite growth and provide protection against toxic stimuli (Previrera et al., 2010, Lu et al., 2015, Takemoto et al., 2015). These secreted factors can be utilised in *in vitro* experiments through the collection of culture media from astrocytes – termed conditioned media; CM – which has been shown to protect neurons against various noxious stimuli including glutamate-induced toxicity (Lu et al., 2015), corticosterone-induced damage (Zhu et al., 2006), methylmercury induced neurotoxicity (Takemoto et al., 2015) and damage induced by reactive oxygen and nitrogen species (Tanaka et al., 1999). In addition, previous work has demonstrated that stimulation of astrocytic β_2 adrenoceptors increases neurite outgrowth and complexity, an effect attributed to the release of trophic factors from astrocytes including FGF-2, GDNF, IL-6, BDNF and NGF- β (Day et al., 2014).

In addition to providing protection against damaging and atrophic stimuli, compelling evidence also demonstrates a critical involvement of astrocytes in synapse formation and function both *in vitro* and *in vivo* (Ullian et al., 2004, Eroglu and Barres, 2010, Allen, 2013, Chung et al., 2013). Early *in vitro* studies observed that retinal ganglion cells (RGCs) in culture form few synapses in the absence of astrocytes (Pfrieger and Barres, 1997), and later studies demonstrated that CM derived from astrocytes was sufficient to induce synaptogenesis (Nägler et al., 2001), suggesting that the synaptogenic effect of astrocytes is mediated through the secretion of soluble factors rather than solely through contact-mediated signalling. Such factors have been found to include ECM proteins thrombospondins (Christopherson et al., 2005), as well as cholesterol (Mauch et al., 2001, Pfrieger, 2003) and TNF α (Beattie et al., 2002). Recent data have also demonstrated an important role for astrocytes in synapse elimination in the developing and adult brain (Chung et al., 2013), suggesting that astrocytes not only aid synapse formation, but also act to refine and modulate synaptic plasticity throughout life.

The re-evaluation of the role of astrocytes in dendrite morphology and synapse formation corresponds to increasing data in the literature which link astrocytic dysfunction to several disease states involving neuronal atrophy and synaptic dysconnectivity including Alzheimer's and

Parkinson's disease (Maragakis and Rothstein, 2006, Verkhatsky et al., 2010, Halliday et al., 2011) chronic pain (Watkins and Maier, 2003, Scholz and Woolf, 2007, Gao and Ji, 2010) ischemic stroke (Anderson et al., 2003, Panickar and Norenberg, 2005, Takano et al., 2009) and psychiatric illnesses such as depression (Cotter et al., 2001, Rajkowska and Miguel-Hidalgo, 2007). In the current investigation, the effects of inducing a specific astrocytic dysfunction using the toxin, L-alpha aminoadipic acid (L-AAA), on neurite outgrowth and synapse number *in vitro* was investigated. L-AAA is a glutamate analogue and occurs naturally in the brain as an intermediate of lysine metabolism. It is reported to be taken up by astrocytes via the high affinity glutamate Na⁺ dependent transport system and accumulates intracellularly prior to exerting its toxicity (Tsai et al., 1996). Although the mechanism of selective toxicity to astrocytes has not been fully elucidated, early *in vitro* studies have indicated that L-AAA acts by inhibiting the glutamate transporters as well as blocking the astrocyte specific enzymes glutamine synthetase and γ -glutamylcysteine synthetase (Olney et al., 1980, McBean, 1994, Brown and Kretschmar, 1998). Glutamine synthetase is an astrocytic enzyme responsible for the conversion of glutamate to glutamine while γ -glutamylcysteine synthetase is responsible for catalysing the first step in the synthesis of glutathione, an important antioxidant in cells. Their inhibition by L-AAA therefore, could lead to glutamate accumulation and reduced glutathione levels in astrocytes respectively. More recent data has shown that prolonged (>72 h) exposure of astrocytes to L-AAA (1 mM) induces apoptosis through the inhibition of protein synthesis (Nishimura et al., 2000).

L-AAA has received particular attention in recent years as an effective method of inducing astrocyte ablation in animal models of depression (Banasr and Duman, 2008, Domin et al., 2014, Lima et al., 2014). L-AAA-induced astrocytic ablation in the prelimbic cortex (PLC) is sufficient to induce depression-like behaviour in rodents, supporting the hypothesis that astrocyte loss contributes to the core symptoms of this illness (Banasr and Duman, 2008). Since depression is also associated with neuronal atrophy as well as spine loss and synaptic deficits, it is of interest to investigate whether astrocyte dysfunctions may contribute towards neuronal deficits in illnesses such as depression. In this regard, a recent study showed that L-AAA-induced astrocyte loss in rats was accompanied by a progressive neuronal loss and dendritic atrophy in the lesion site of the mPFC, suggesting that astrocytic dysfunction creates knock-on effects for neuronal integrity which may result in the observed behavioural and cognitive impairments (Lima et al., 2014).

In this investigation, we aimed to firstly characterise the temporal and concentration dependent effects of L-AAA on the viability of astrocytes, microglia and neurons to establish the specificity of L-AAA as an astrocyte toxin. To further characterise the mode of toxicity to astrocytes we investigated the effects of L-AAA on mRNA expression of markers related to glutamate transport and inflammation, as well as assessing its effects on astrocytic oxygen consumption rates as a measure of mitochondrial function. Following on from this, we were particularly interested in

investigating the effects of L-AAA-induced astrocytic dysfunction on neurite outgrowth and synapse number in primary cortical neurons. We employed the use of an *in vitro* model consisting of astrocyte CM to investigate soluble factors derived from L-AAA treated astrocytes on neurite outgrowth and synapse number. Finally, we examined the efficacy of targeting the neuronal NMDA-R/NO signalling pathway as a strategy of promoting neurotrophic effects following L-AAA CM induced atrophy based on reports indicating that low concentrations of NMDA-R antagonists possess neurotrophic properties (Robson et al., 2012, Brown et al., 2015).

5.2 Study Designs

5.2.1 Characterisation of the effects of L-AAA on viability of primary cell cultures

The specificity of L-AAA as an astroglial toxin was investigated by assessing the effects of L-AAA (0.05, 0.5 and 5 mM) on different cell types *in vitro*. Primary cortical mixed glia, purified astrocytes, microglia (DIV 13) and neurons (DIV 3) were treated with L-AAA for 24 h and 48 h. Cell viability was assessed using the Alamar blue test - a widely used fluorometric assay which is based on the ability of viable cells to convert resazurin to highly fluorescent resorufin via mitochondrial metabolic enzymes (Rampersad, 2012). Concentrations of L-AAA were chosen based on prior research which used a similar range of concentrations on astrocytes (Brown and Kretzschmar, 1998, Nishimura et al., 2000).

The potential for recovery in L-AAA treated astrocytes was also examined to investigate whether L-AAA induced a transient or permanent toxicity to astrocytes *in vitro*. For this study, astrocytes were treated for 48 h with L-AAA (5 mM) and subsequently provided with fresh culture media for an additional 48 h before re-evaluation of cell viability using Alamar blue.

Finally, since L-AAA is a glutamate analogue, primary cortical astrocytes (DIV 13) were treated with glutamate at the same concentrations (0.05, 0.5 and 5 mM) for 24 h or 48 h to assess whether L-AAA mediated changes generalise to glutamate. Cell viability was assessed using Alamar blue.

5.2.2 Characterisation of the effects of L-AAA on astrocyte mRNA expression profiles

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05, 0.5 and 5 mM) for 24 h. Cells were harvested for RNA extraction followed by RT-PCR and mRNA expression was assessed for the astroglial markers, glial fibrillary acidic protein (GFAP) and S100B, the glutamate transporters, GLT1 and GLAST, inflammatory markers, tumor necrosis factor- α (TNF α) and iNOS, the apoptotic marker caspase-3 and the enzyme responsible for L-AAA degradation, kynurenine/alpha-aminoadipate aminotransferase (KATII/AADAT). KATII is preferentially expressed in astrocytes in the brain (Vécsei et al., 2013), and L-AAA-induced changes in KATII may be indicative of impaired metabolism and accumulation of the toxin in astrocytes.

5.2.3 Effects of L-AAA on mitochondrial respiration

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05, 0.5 and 5 mM) for 24 h and the oxygen consumption rate (OCR) was assessed as an indicator of mitochondrial respiration and cellular function using the Extracellular Flux Analyzer (Seahorse Biosciences).

5.2.4 Effects of astrocyte CM on neurite outgrowth

Primary cortical astrocytes (DIV 13) were provided with fresh media for 24 h or 48 h and the resulting media (termed conditioned media; CM) was applied to primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry for Sholl analysis.

5.2.5 Characterisation of the effects of astrocyte L-AAA CM on neurite outgrowth

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05, 0.5 and 5 mM) for 24 h or 48 h and the resulting L-AAA CM was applied to primary cortical neurons (DIV 3) for 24 h or 48 h before fixation and immunocytochemistry for Sholl analysis (**Figure 5.1**).

In addition to CM studies, neurons were treated directly with L-AAA in order to assess whether L-AAA impacted upon neurite outgrowth alone. For this experiment, primary cortical neurons (DIV 3) were treated for 48 h with L-AAA before fixation and immunocytochemistry for Sholl analysis.

Finally, the ability of NMDA-R/NO signalling pathway modulation was assessed as a strategy to protect neurons from L-AAA CM-induced neurite regression. Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05, 0.5 and 5 mM) for 24 h. Primary cortical neurons (DIV 3) were co-treated with L-AAA CM in addition to ketamine (100 nM), IC87201 (100 nM) or TRIM (100 nM) for 24 h before fixation and immunocytochemistry for Sholl analysis. Concentrations of ketamine were chosen based on prior reports indicating its ability to potentiate NGF-induced neurite outgrowth in PC12 cells at similar concentrations, (Robson et al., 2012) and TRIM was chosen at this concentration for direct comparison. For IC87201, previous published reports indicated that IC87201 dose-dependently reduced NMDA-induced cGMP production in primary hippocampal neurons (DIV 14-21) with an IC₅₀ of 2.7 μ M (Florio et al., 2009).

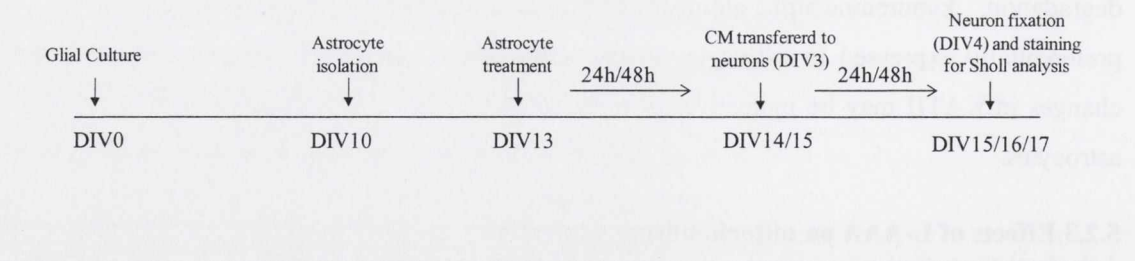


Figure 5.1: Experimental timeline for Sholl experiments involving L-AAA CM. Primary mixed glia were cultured until confluent (DIV 10) before astrocyte isolation. Purified astrocytes were treated for 24 h or 48 h with L-AAA and the resulting CM from these astrocytes (termed L-AAA CM) was transferred onto primary cortical neurons (DIV 3) for 24 h or 48 h before fixation and immunocytochemistry for Sholl analysis.

5.2.6 Characterisation of the effects of NMDA-R/NO signalling inhibition on astrocytic L-AAA CM-induced reductions the co-localised expression of synaptophysin and PSD-95

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05 and 0.5 mM) for 24 h and the resulting CM from these astrocytes was applied to mature primary cortical neurons (DIV 20) for 24 h with/without co-treatment with ketamine, TRIM or IC87201 (100 nM), before fixation and immunocytochemistry for quantification of the number of co-localised synaptophysin and PSD-95 puncta as described in Chapter 2: Materials and Methods.

5.3: Results

5.3.1 Characterisation of the effects of L-AAA on the viability of primary cell cultures

The specificity of L-AAA as an astrocytic toxin was investigated by treating mixed glia, purified astrocytes, purified microglia and neurons with L-AAA. Viability was assessed using the Alamar blue viability test after 24 h and 48 h of treatment with L-AAA for each cell type. A subset of cells were fixed and immunostained to confirm the expression of cell specific markers. Primary mixed glial cells, purified astrocytes and purified microglia were immunostained for the astrocyte marker, GFAP, the microglial marker, Cd11b, and the DAPI nuclear stain. Neuronal cultures were stained with the neuronal specific marker, β III tubulin and DAPI to identify non-neuronal cells (Figure 5.2A-D).

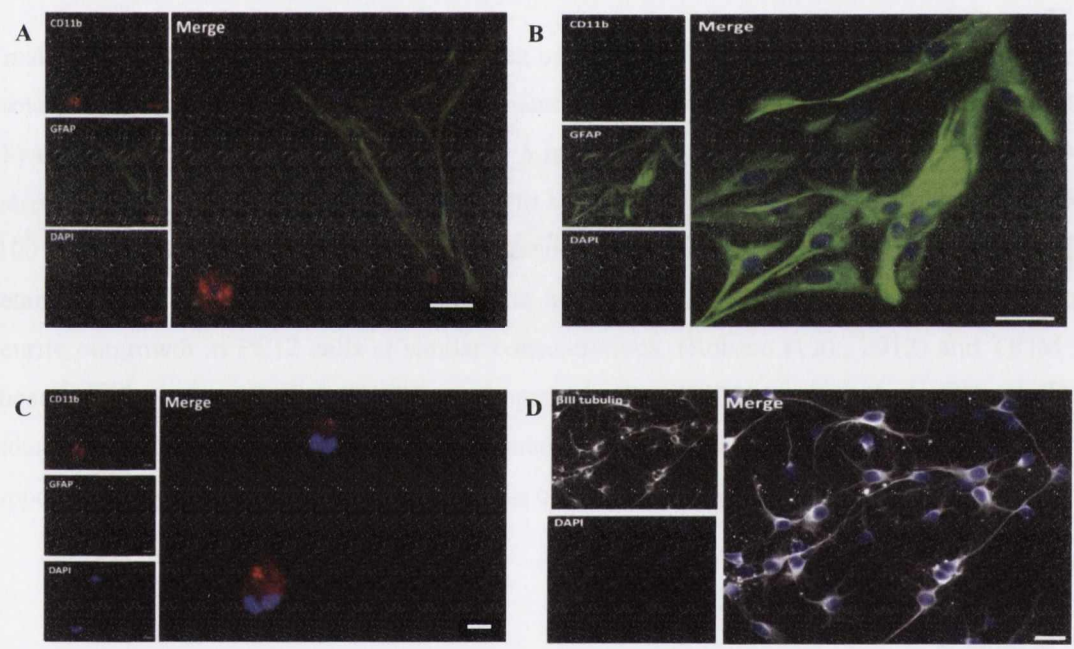


Figure 5.2: Representative images of primary cell cultures. Primary mixed glial cultures (A), primary purified astrocytes (B) and primary purified microglia (C) were fixed at DIV 13 and stained with the microglial marker CD11b, the astrocyte marker GFAP and the DAPI nuclear stain. Scale bar = 20 μ m. Primary purified neurons (DIV 3) (D) were fixed and stained with the neuronal specific marker β -III tubulin (white) and the DAPI nuclear stain (blue) to identify any non-neuronal cells. Scale bar = 50 μ m.

5.3.1.1: Effects of L-AAA on primary mixed glial viability

One-way ANOVA failed to demonstrate effects of L-AAA on the viability of mixed glial cultures (DIV 13) after 24 h of treatment. In contrast, one way ANOVA demonstrated an effect of L-AAA on mixed glia after 48 h ($F_{3,59}=25.06$, $p<0.001$). *Post hoc* analysis revealed a decrease in mixed glial viability after 48 h with L-AAA at a concentration of 5 mM ($p<0.001$) (**Figure 5.3A**).

5.3.1.2: Effects of L-AAA on primary purified astrocyte viability

Since mixed glial cultures are composed of both astrocytes and microglia, we investigated the effects of L-AAA firstly on purified astrocytes (DIV 13). Similar to the effects observed with mixed glia, one-way ANOVA demonstrated that L-AAA has no effects on astrocyte viability after 24 h of treatment. In contrast, one way ANOVA demonstrated an effect of L-AAA on astrocytes after 48 h of treatment ($F_{3,57}=21.21$, $p<0.001$). *Post hoc* analysis revealed a decrease in astrocyte viability after 48 h with L-AAA at a concentration of 5 mM ($p<0.001$) (**Figure 5.3B**).

5.3.1.3: Effects of L-AAA on primary purified microglial viability

One-way ANOVA failed to demonstrate effects of L-AAA on the viability of primary purified microglia (DIV 13) after 24 h of treatment or 48 h of treatment (**Figure 5.3C**).

5.3.1.4: Effects of L-AAA on primary purified neuronal viability

Primary neurons (DIV 3) were also treated with L-AAA. One way ANOVA failed to demonstrate effects of L-AAA on primary cortical neurons after 24 h of treatment or 48 h of treatment (**Figure 5.3D**).

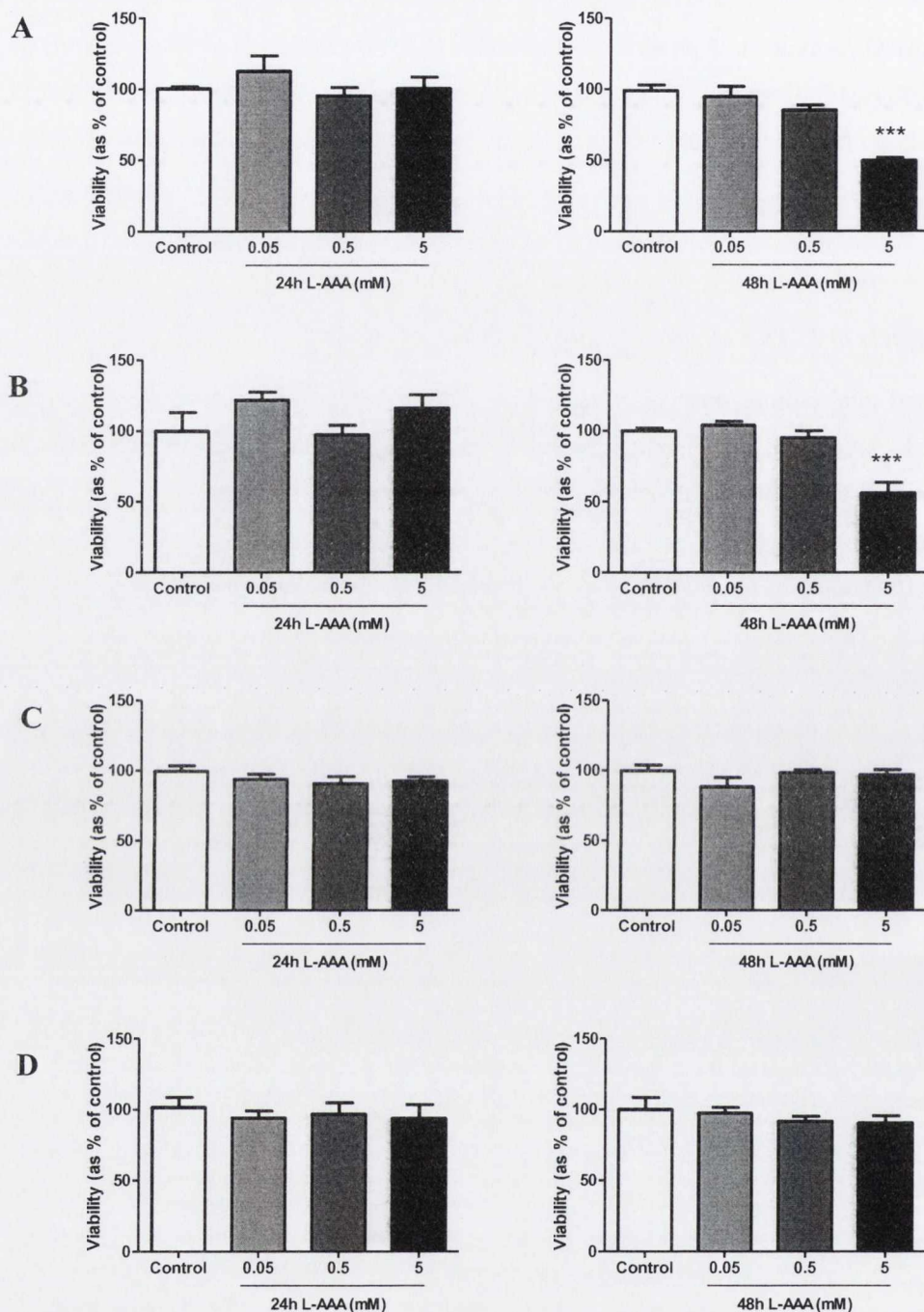


Figure 5.3: Effects of L-AAA on the viability of primary cell cultures. Primary mixed glial cultures (A), primary purified astrocytes (B) and primary purified microglia (C) were treated at DIV 13 with L-AAA for 24 h or 48 h. Purified neuronal cultures (DIV 3) (D) were also treated with L-AAA for 24 h or 48 h. Viability was assessed using the Alamar blue test. Data is expressed as mean $\pm$ SEM, $n=15-19$ wells, representative of 3 independent experiments *** $p<0.001$ vs. Control (Student-Newman-Keuls).

5.3.1.5: Effects of a recovery period on L-AAA-induced decreases in astrocyte viability

The ability of astrocytes to recover from L-AAA-induced reductions in viability was examined by testing cell viability after 48 h of treatment and subsequently providing astrocytes with fresh media for a following 48 h ‘recovery’ period before re-assessment. Control-treated astrocytes were also provided with fresh media. Two-way repeated measures ANOVA demonstrated an effect of L-AAA treatment ($F_{1,30}=38.09$, $p<0.001$), but failed to demonstrate an effect of time ($F_{1,30}=0.27$, $p=0.61$), or a L-AAA x time interaction ($F_{1,30}=21$, $p=0.65$) on the viability of primary cortical astrocytes. *Post hoc* analysis revealed that astrocytes treated with L-AAA (5 mM) showed reductions in viability after 48 h ($P<0.01$), and this reduction in viability was maintained for 48 h as evidenced by a decrease in viability between control astrocytes and astrocytes treated with L-AAA (5 mM) and provided with a 48 h recovery period ($P<0.01$) (Figure 5.4).

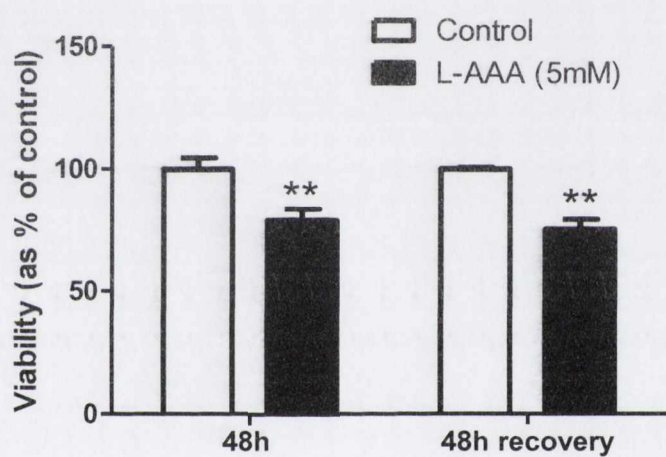


Figure 5.4: Effects of L-AAA on astrocyte viability after a recovery period of 48 h. Primary cortical astrocytes (DIV 13) were treated with L-AAA for 48 h, and then provided with fresh media for a recovery period of 48 h. Viability was assessed using the Alamar Blue test. Data is expressed as mean $\pm$ SEM, $n=13-16$ wells, representative of 3 independent experiments ** $p<0.05$ vs. respective Control (Student-Newman-Keuls).

5.3.1.6: Effects of glutamate on astrocyte viability

Since L-AAA is a glutamate analogue, the effect of glutamate on primary cortical astrocytic viability was investigated. This was carried out in order to assess whether L-AAA-induced reductions in viability generalised to compounds of similar structure rather than being an L-AAA-specific effect. A one-way ANOVA demonstrated an effect of glutamate on astrocyte viability after 24 h of treatment ($F_{3,60}=9.87$, $p<0.001$) but failed to demonstrate effects after 48 h of treatment. *Post hoc* analysis revealed that the treatment of astrocytes for 24 h with glutamate at 0.05 mM and 0.5 mM increases astrocyte viability compared to control-treated astrocytes ($p<0.01$ and $p<0.001$ respectively) (Figure 5.5).

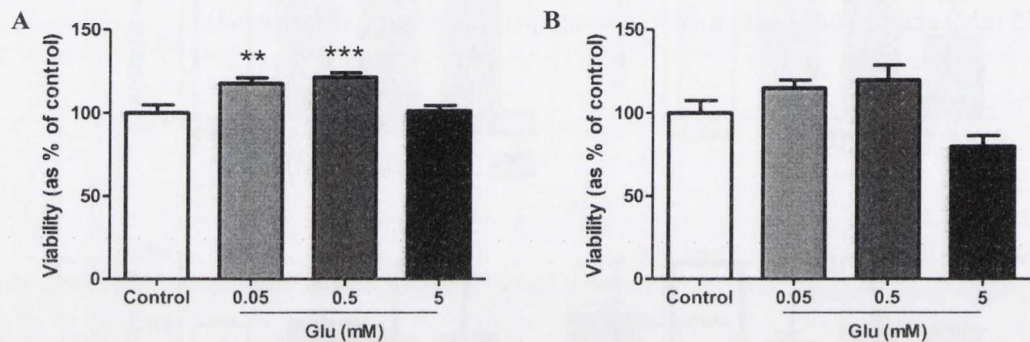


Figure 5.5: Effects of glutamate on the viability of astrocytes. Purified primary astrocytes (DIV 13) were treated for 24 h (A) or 48 h (B) with glutamate (0.05 - 5 mM). Viability was assessed with the Alamar Blue test. Data is expressed as mean $\pm$ SEM, $n=12-16$ wells, representative of 3 independent experiments *** $P<0.001$ and ** $P<0.01$ vs. Control (Student-Newman-Keuls).

5.3.2 Characterisation of the effects of L-AAA on the mRNA expression of astrocytic markers

Astrocytes (DIV 13) were treated for 24 h with L-AAA (0.05, 0.5, 5 mM). Cells were harvested for RNA extraction followed by RT-PCR to analyse the expression of astrocyte-specific markers, astrocyte specific glutamate transporters, as well as inflammatory and apoptotic mediators.

5.3.2.1 Effects of L-AAA on mRNA expression of astrocytic markers

One way ANOVA demonstrated an effect of L-AAA on GFAP mRNA expression ($F_{3,23}=43.75$, $p<0.001$). *Post hoc* analysis revealed that astrocytes treated with L-AAA (0.5 mM and 5 mM) showed a decrease in GFAP mRNA expression compared to control-treated astrocytes ($p<0.001$). One way ANOVA also demonstrated an effect of L-AAA on S100B mRNA expression ($F_{3,23}=11.73$, $p<0.0001$). *Post hoc* analysis revealed that astrocytes treated with L-AAA (5 mM) showed an increase in S100B mRNA expression compared to control-treated astrocytes ($p<0.001$) (**Figure 5.6A,B**).

5.3.2.2 Effects of L-AAA on mRNA expression of astrocytic glutamate transporters

One way ANOVA demonstrated an effect of L-AAA on GLT-1 mRNA expression ($F_{3,22}=40.54$, $p<0.001$). *Post hoc* analysis revealed that astrocytes treated with L-AAA (5 mM) showed an increase in GLT-1 mRNA expression compared to control-treated astrocytes ($p<0.001$). In addition, one way ANOVA demonstrated an effect of L-AAA on GLAST mRNA expression ($F_{3,22}=49.42$, $p<0.001$). *Post hoc* analysis revealed that astrocytes treated with L-AAA (0.5 mM and 5 mM) showed a decrease in GLAST mRNA expression compared to control-treated astrocytes ($p<0.001$), and also revealed that astrocytes treated with L-AAA (0.05 mM) demonstrated an increase in GLAST mRNA expression compared to control-treated astrocytes ($p<0.01$) (**Figure 5.6C, D**).

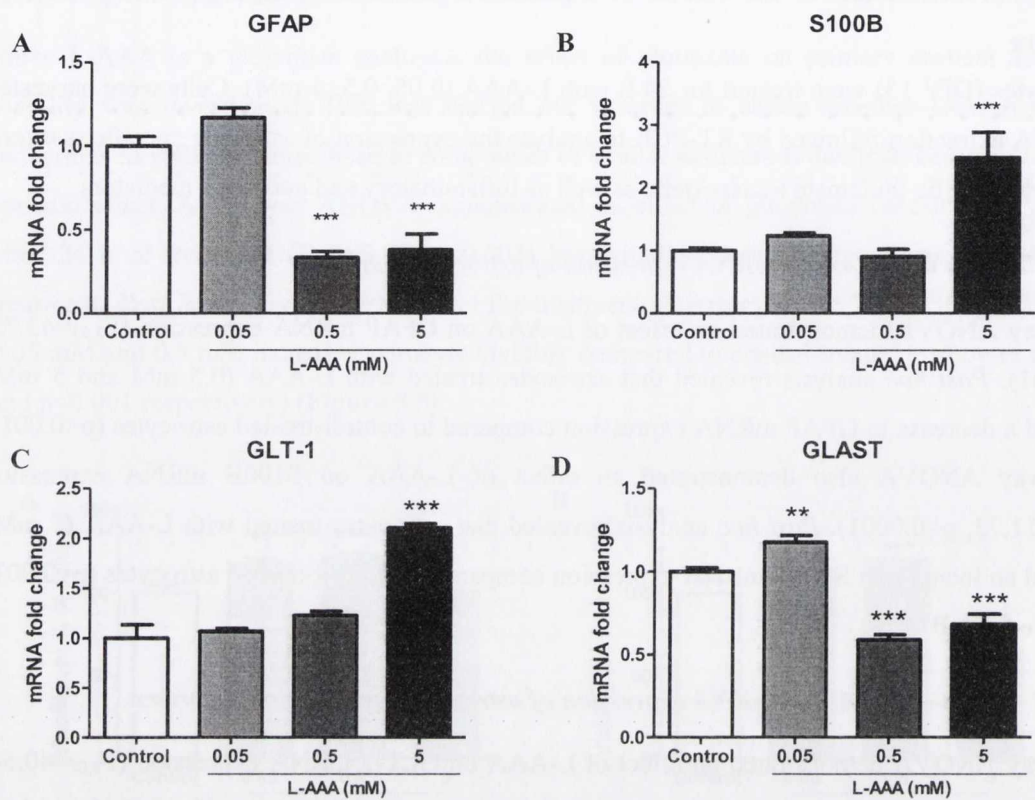


Figure 5.6: Effects of L-AAA on mRNA expression of astrocytic markers and glutamate transporters. Primary cortical astrocytes (DIV 13) were treated for 24 h with L-AAA (0.05, 0.5, 5 mM). Cells were harvested for RNA extraction followed by RT-PCR to analyse the expression of (A) GFAP, (B) S100B, (C) GLT-1 and (D) GLAST. Data are expressed as mean $\pm$ SEM n=6 wells per group ***P<0.001, **P<0.01 vs. Control (Student-Newman-Keuls).

5.3.2.3 Effects of L-AAA on mRNA expression of inflammatory markers, caspase-3 and KATII

One way ANOVA failed to demonstrate effects of L-AAA (0.05-5 mM) on the mRNA expression of inflammatory mediators, TNF α or iNOS. One way ANOVA also failed to demonstrate effects of L-AAA (0.05-5 mM) on the mRNA expression of the apoptosis-related gene, caspase-3. Finally, one way ANOVA failed to demonstrate effects of L-AAA (0.05 - 5 mM) on the mRNA expression of the enzyme responsible for its degradation, KATII (Figure 5.7).

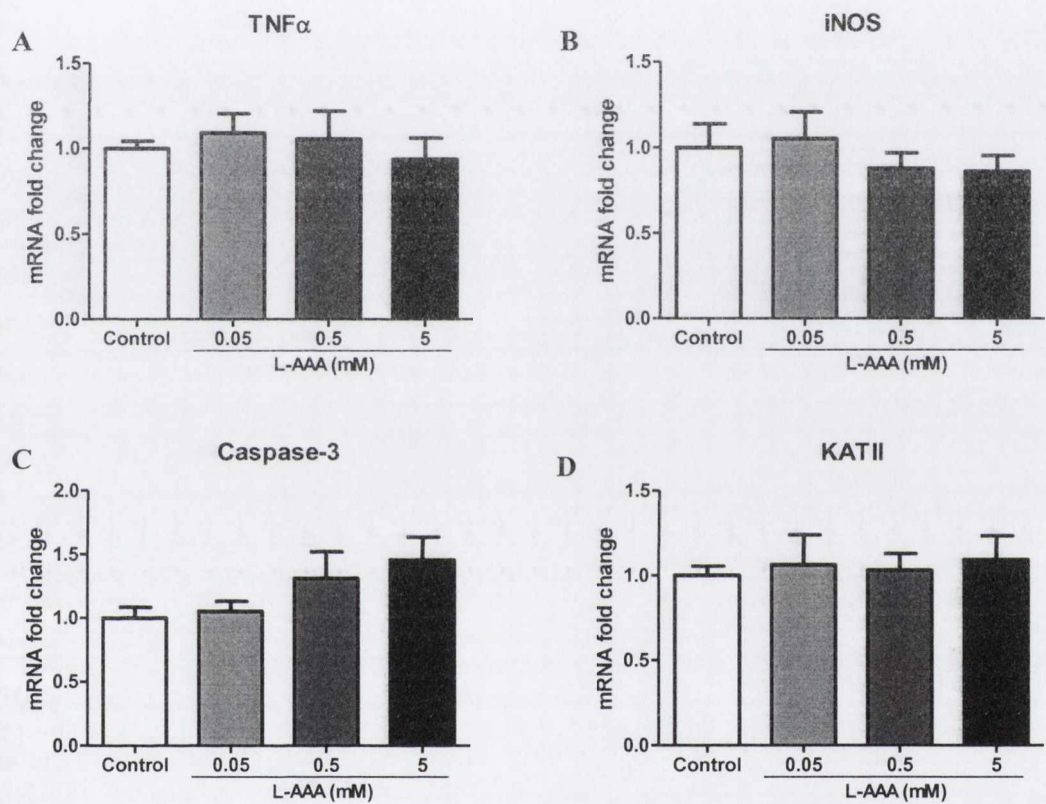


Figure 5.7: Effects of L-AAA on TNF α , iNOS, caspase-3 and KATII mRNA expression. Primary cortical astrocytes (DIV 13) were treated for 24h with L-AAA (0.05, 0.5, 5 mM). Cells were harvested for RNA extraction followed by RT-PCR to analyse the expression of (A) TNF α , (B) iNOS, (C) caspase-3 and (D) KATII. Data are expressed as mean $\pm$ SEM, n=6 wells per group (Student-Newman-Keuls).

5.3.3 Characterisation of the effects of L-AAA on mitochondrial respiration

To further investigate the mechanism of L-AAA-induced astrocyte toxicity we analysed the oxygen consumption rate (OCR) as an indicator of mitochondrial respiration using an Extracellular Flux Analyzer (Seahorse Biosciences). Astrocytes were treated with L-AAA for 24 h. One way ANOVA demonstrated an effect of L-AAA on the OCR ($F_{3,12}=10.23$, $p=0.001$). *Post hoc* analysis revealed that astrocytes treated for 24h with L-AAA (0.5 and 5 mM) had reduced OCR compared to control-treated neurons ($P<0.05$ and $P<0.01$) (Figure 5.8).

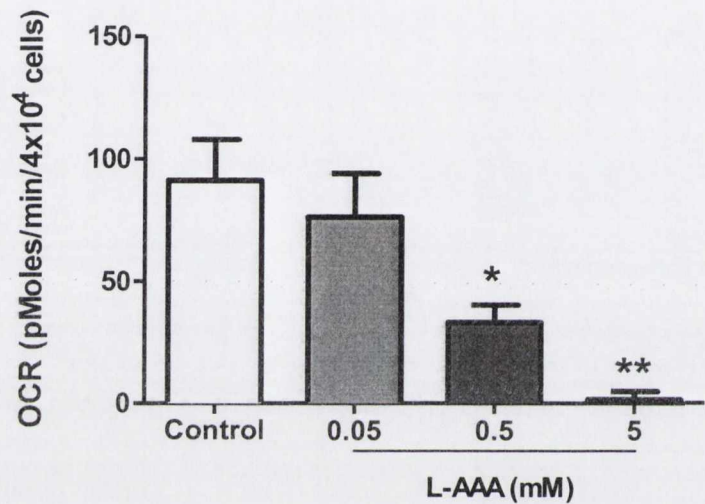


Figure 5.8: Effects of L-AAA on oxygen consumption rates. Primary cortical astrocytes (DIV 13) were treated for 24 h with L-AAA (0.05, 0.5 and 5 mM) and the oxygen consumption rate (OCR) was measured using an Extracellular Flux Analyzer (Seahorse Biosciences). Data are expressed as mean $\pm$ SEM, $n=5$ wells per group ** $P<0.01$, * $P<0.05$ vs. Control (Student-Newman-Keuls).

5.3.4 Effects of astrocyte CM on neurite outgrowth

Since astrocytes have been shown to aid neuronal growth (Previtera et al., 2010), and CM derived from astrocytes have been reported to protect neurons against several deleterious stimuli (Zhu et al., 2006, Takemoto et al., 2015), we investigated whether astrocyte CM was sufficient to increase neurite outgrowth in primary cortical neurons. Fresh culture media was provided to astrocytes for either 24 h or 48 h and the resulting astrocyte CM was subsequently transferred to primary cortical neurons (DIV 3) for 24 h. Control-treated neurons were provided with standard Neurobasal culture media.

A Student's *t*-test demonstrated an effect of 24 h astrocyte CM on the number of primary neurites ($t=3.84$, $df=36$, $p<0.01$) and the number of neuritic branches ($t=2.04$, $df=41$, $p<0.01$) compared to control neurons. Two-way repeated measures ANOVA demonstrated an effect of CM ($F_{1,627}=11.03$, $p<0.01$), an effect of distance ($F_{19,627}=7051$, $p<0.01$) and a treatment x distance interaction ($F_{19,627}=3.89$, $p<0.01$) on the number of neuritic branches at specific distances from the neuronal cell soma. *Post hoc* analysis revealed that neurons treated with CM had significantly more branches than control-treated neurons at 20 μm ($P<0.05$) and at 30-50 μm ($P<0.01$) (**Figure 5.9A**).

A Student's *t*-test demonstrated an effect of 48 h astrocyte CM on the number of primary neurites ($t=5.43$, $df=56$, $p<0.01$), neuritic length ($t=3.54$, $df=47$, $p<0.01$) and the number of neuritic branches ($t=2.78$, $df=47$, $p<0.01$) compared to control neurons. Two-way repeated measures ANOVA demonstrated an effect of CM ($F_{1,456}=16.9$, $p<0.01$), an effect of distance ($F_{19,456}=263.2$, $p<0.01$) and a treatment x distance interaction ($F_{19,456}=2.36$, $p<0.01$) on the number of neuritic branches at specific distances from the neuronal cell soma. *Post hoc* analysis revealed that neurons treated with CM had significantly more branches than control-treated neurons at 10-20 μm ($P<0.01$) (**Figure 5.9B**).

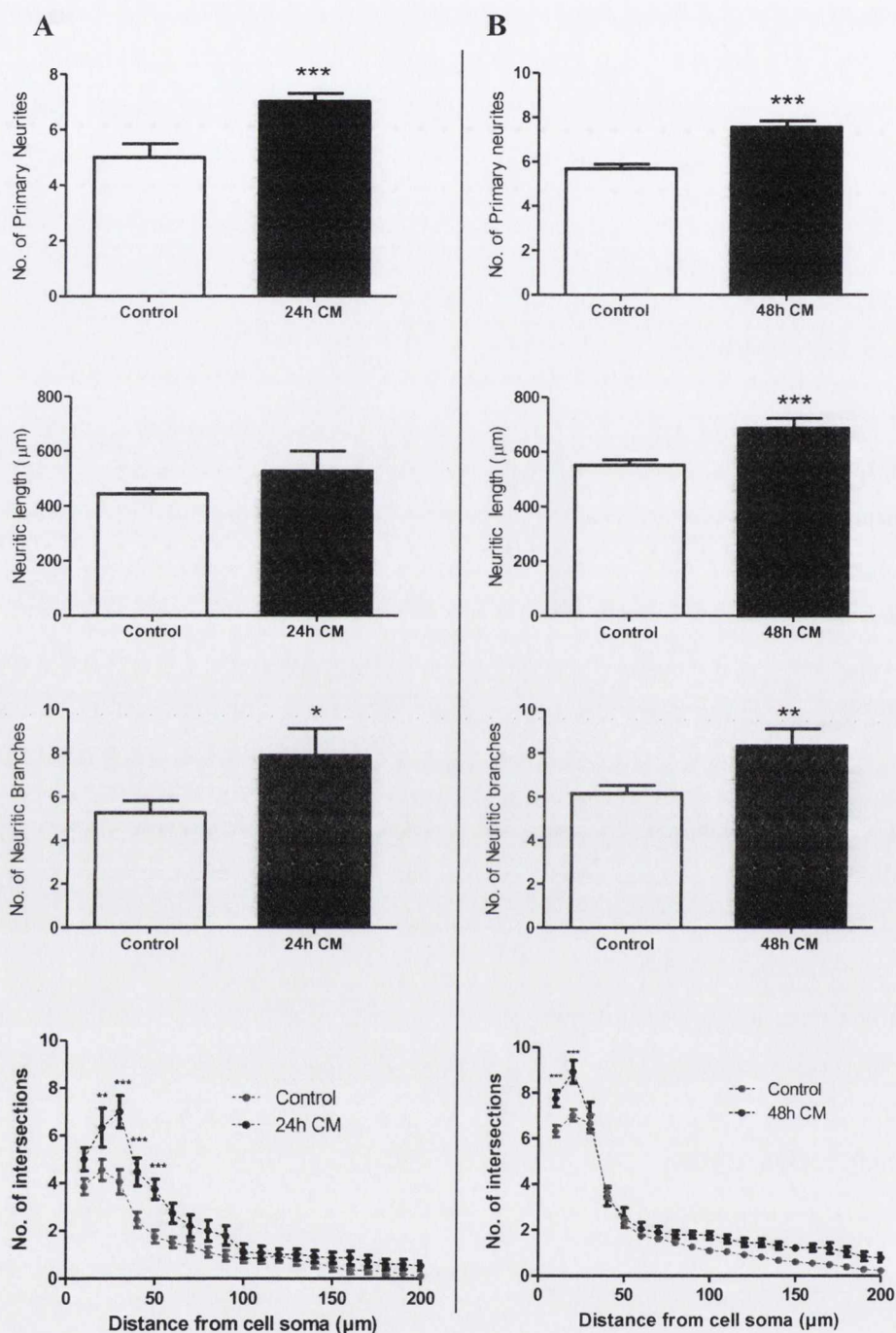


Figure 5.9: Effect of astrocyte conditioned media (CM) on neurite outgrowth. Astrocytes (DIV 13) were provided with fresh culture media for 24 h (A) or 48 h (B) and the resulting astrocyte conditioned media (CM) was transferred to primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry. Sholl analysis was performed on neurons to examine the number of primary neurites, the neuritic length and the Sholl profile. Data are expressed as mean $\pm$ SEM, $n=20-30$ coverslips per group representative of 3 independent experiments *** $P<0.001$ ** $P<0.01$ and * $P<0.05$ vs. Control; Sholl profile, *** $P<0.001$ and ** $P<0.01$ CM vs. Control (Neurobasal media) (Student-Newman-Keuls).

5.3.5 Characterisation of the effects of media derived from L-AAA-treated astrocytes (L-AAA CM) on neurite outgrowth

Considering CM derived from untreated astrocytes increased several measures of neuronal outgrowth, we subsequently investigated the effects of CM derived from L-AAA-treated astrocytes (L-AAA CM) on neuronal outgrowth and complexity. Astrocytes (DIV 13) were treated for 24 h or 48 h with L-AAA and the media from these cells (24 h L-AAA CM or 48 h L-AAA CM) was transferred to primary cortical neurons (DIV 3) for 24 h or 48 h.

5.3.5.1 Effects of media derived from astrocytes treated with L-AAA for 24h (24h L-AAA CM) on neurite outgrowth

24 h neuronal treatment

Astrocytes were treated for 24 h with L-AAA and the resulting L-AAA CM was applied to primary cortical neurons for 24 h. One-way ANOVA demonstrated an effect of L-AAA CM on the neuritic length ($F_{2,68}=5.227$, $P=0.007$) and the number of neuritic branches ($F_{2,61}=2.61$, $P=0.02$) but no effect on the number of primary neurites extending from the cell soma ($F_{2,68}=0.8591$, $P=0.43$). *Post hoc* analysis revealed that 24 h L-AAA CM (5 mM) decreased the neuritic length ($P<0.01$) and number of number of neuritic branches ($p<0.05$) when compared to control CM-treated neurons. Furthermore, a two-way repeated measures ANOVA demonstrated an effect of 24 h L-AAA CM treatment ($F_{2,1273}=5.1$, $P=0.009$) on the number of neuritic branches at specific distances from the cell soma. ANOVA also demonstrated an effect of distance ($F_{19,1273}=7.35$, $p<0.001$) and a treatment x distance interaction ($F_{38,1273}=2.45$, $p<0.001$). *Post hoc* analysis revealed that neurons treated for 24 h with both concentrations of L-AAA CM (0.5 and 5 mM) had fewer branches than control-treated neurons at 30 μm ($P<0.001$), while neurons treated with 5 mM L-AAA CM had fewer branches at 20 μm ($P<0.05$) (**Figure 5.10A**).

48 h neuronal treatment

Astrocytes were treated for 24 h with L-AAA and the resulting L-AAA CM was applied to primary cortical neurons for 48 h. One-way ANOVA demonstrated an effect of L-AAA CM on the neuritic length ($F_{2,38}=5.3$, $P=0.01$), the number of neuritic branches ($F_{2,44}=3.581$, $P=0.036$), and the number of primary neurites extending from the cell soma ($F_{2,58}=23.51$, $P<0.01$). *Post hoc* analysis revealed that L-AAA CM (5 mM) decreased the neuritic length and the number of neuritic branches ($p<0.05$) while L-AAA (0.5 and 5 mM) decreased the number of primary neurites when compared to control CM-treated neurons. Two-way repeated measures ANOVA demonstrated an effect of distance ($F_{19,817}=108.5$, $p<0.001$) on the number of neuritic branches at specific distances from the neuronal cell soma but failed to demonstrate effects of L-AAA CM

treatment ($F_{2,817}=10.16$, $p=0.61$) or a treatment x distance interaction ($F_{38,817}=0.55$, $p=0.99$) (Figure 5.10B).

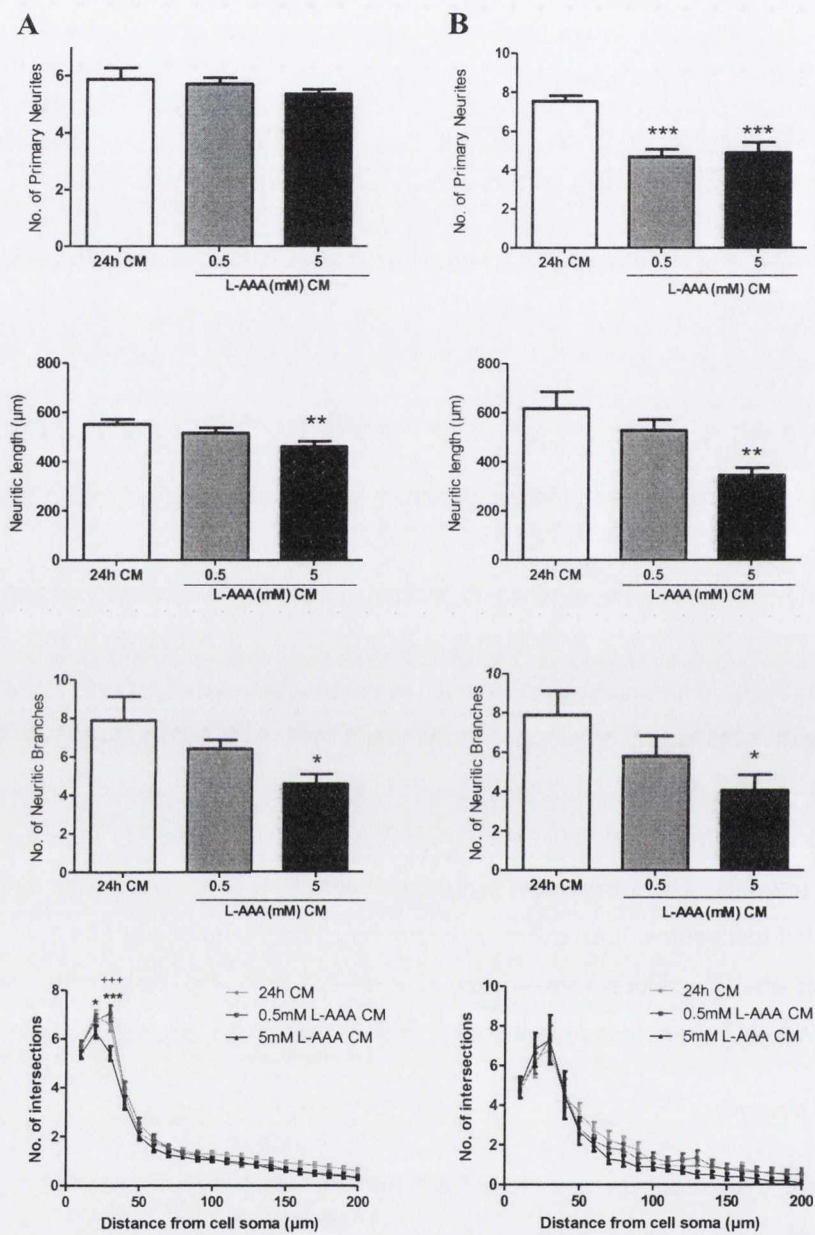


Figure 5.10: Effects of 24 h L-AAA CM on neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h (A) or 48 h (B) with CM from untreated astrocytes (24 h CM) or CM from astrocytes treated with L-AAA for 24 h (24 h L-AAA CM). Immunocytochemistry was performed on neurons at DIV 4 or DIV 5. Neurons were identified using the neuronal structural protein β -III tubulin and the DAPI nuclear stain with fluorescence microscopy. Sholl analysis was performed on neurons to examine the number of primary neurites, the neuritic length, the number of neuritic branches and the Sholl profile. Data are expressed as mean $\pm$ SEM, $n=20$ -30 coverslips per group representative of 3 independent experiments. *** $P<0.001$, ** $P<0.01$ and * $P<0.05$ vs. 24 h CM; Sholl profile, *** $P<0.001$ and * $P<0.05$ L-AAA (5 mM) CM vs. 24 h CM, +++ $P<0.001$ L-AAA (0.5 mM) CM vs. L-AAA (5 mM) CM (Student-Newman-Keuls).

5.3.5.2 Effects of media derived from astrocytes treated with L-AAA for 48 h (48 h L-AAA CM) on neurite outgrowth

24 h neuronal treatment

Astrocytes were treated for 48 h with L-AAA and the resulting L-AAA CM was applied to primary cortical neurons for 24 h. One-way ANOVA demonstrated an effect of 48 h L-AAA CM on the number of primary neurites ($F_{2,49}=7.830$, $P<0.001$), the neuritic length ($F_{2,49}=13.62$, $P<0.001$), and the number of neuritic branches ($F_{2,50}=7.703$, $P<0.001$). *Post hoc* analysis revealed that 48 h L-AAA CM (0.5 mM and 5 mM) decreased the number of neuritic branches and neuritic length ($p<0.05$ and $p<0.001$ respectively) when compared to control CM-treated neurons. In addition, neurons treated with L-AAA CM (5 mM) had fewer neuritic branches ($p<0.05$) and reduced neuritic length ($p<0.01$) compared to the lower concentration of L-AAA CM (0.5 mM). With regard to the Sholl profile, a two-way repeated measures ANOVA demonstrated an effect of 48 h L-AAA CM treatment ($F_{2,931}=11.33$, $p<0.0001$) on the number of neuritic branches at specific distances from the neuronal cell soma. ANOVA also demonstrated an effect of distance ($F_{19,931}=498.9$, $p<0.0001$) and a treatment x distance interaction ($F_{38,931}=1.58$, $p=0.02$). *Post hoc* analysis revealed that neurons treated for 48 h with both concentrations of L-AAA CM (0.5 and 5 mM) had fewer branches than control CM-treated neurons at 10 μm and 20 μm ($P<0.01$ and $P<0.001$), and neurons treated with 5 mM L-AAA CM had fewer branches than the lower concentration of 0.5 mM L-AAA CM at 30 μm ($P<0.05$) (**Figure 5.11A**).

48 h neuronal treatment

Astrocytes were treated for 48 h with L-AAA and the resulting L-AAA CM was applied to primary cortical neurons for 48 h. One-way ANOVA demonstrated an effect of 48 h L-AAA CM on the neuritic length ($F_{2,36}=11.59$, $P<0.001$) and on the number of neuritic branches ($F_{2,37}=9.24$, $P<0.001$) but failed to demonstrate effects on the number of primary neurites. *Post hoc* analysis revealed that 48 h L-AAA (5 mM) CM decreased both the number of neuritic branches and the neurite length compared to control-treated neurons ($p<0.01$) and compared to neurons treated with L-AAA (0.5 mM) CM. L-AAA (0.5 mM) CM also decreased the neuritic length relative to control-treated neurons. In terms of the Sholl profile, two-way repeated measures ANOVA demonstrated an effect of 48 h L-AAA CM treatment ($F_{2,1900}=3.03$, $p=0.05$) on the number of neuritic branches at specific distances from the neuronal cell soma. ANOVA also demonstrated an effect of distance ($F_{19,1900}=234.9$, $p<0.0001$), but failed to demonstrate a treatment x distance interaction ($F_{38,1900}=0.95$, $p=0.5$). *Post hoc* analysis revealed that neurons treated for 48 h with L-AAA CM (5 mM) had fewer branches than control-treated neurons at 40 μm ($P<0.001$) (**Figure 5.11B**).

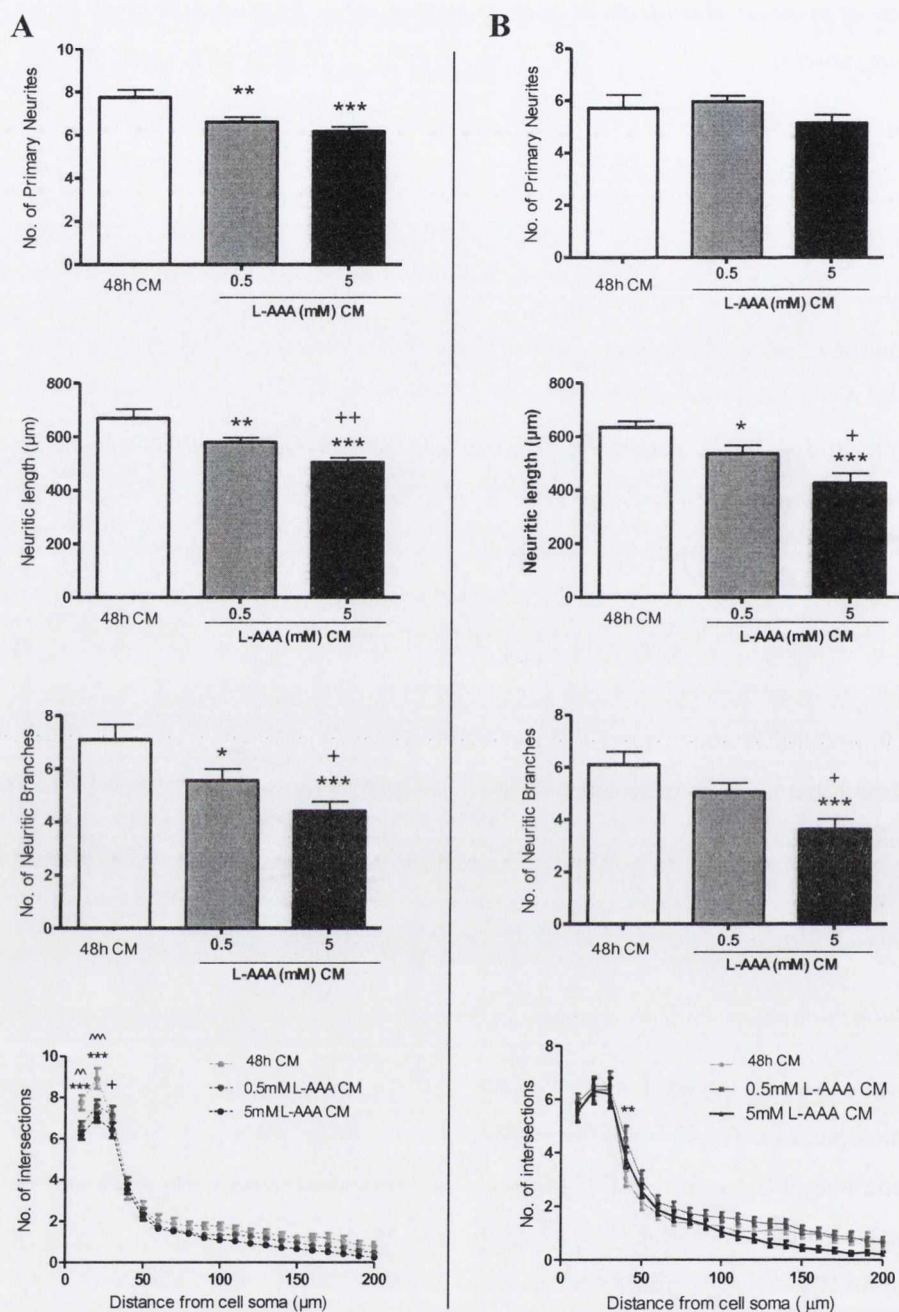


Figure 5.11 Effects of 48 h L-AAA CM on neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h (A) or 48 h (B) with CM from untreated astrocytes (48 h CM) or CM from astrocytes treated with L-AAA for 48 h (48 h L-AAA CM). Immunocytochemistry was performed at DIV 4 or DIV 5. Neurons were identified using the neuronal structural protein β -III tubulin and the DAPI nuclear stain with fluorescence microscopy. Sholl analysis was performed on neurons to examine the number of neuritic branches, neuritic length, primary neurites and Sholl profile. Data are expressed as mean $\pm$ SEM, $n=18$ -20 coverslips per group representative of 3 independent experiments *** $P<0.001$ ** $P<0.01$ and * $P<0.05$ L-AAA CM vs. 48 h CM, ++ $P<0.01$ and + $P<0.05$ L-AAA (5 mM) CM vs. L-AAA (0.5 mM) CM; Sholl profile, *** $P<0.001$ ** $P<0.01$ L-AAA (5 mM) vs. 48 h CM, + $P<0.05$ L-AAA (0.5 mM) CM vs. 48 h Ctrl CM, ^^ $P<0.001$ and ^^ $P<0.01$ L-AAA (5 mM) vs. L-AAA (0.5 mM) (Student-Newman-Keuls).

5.3.5.3 Direct treatment of primary cortical neurons with L-AAA for 48 h

One-way ANOVA failed to demonstrate effects of L-AAA (0.5 and 5 mM) on the number of primary neurites extending from the cell soma ($F_{2,38}=0.03$, $p=0.97$), the neuritic length ($F_{2,57}=0.26$, $p=1.39$), or the number of neuritic branches ($F_{2,38}=1.17$, $p=0.32$). Furthermore, two-way ANOVA demonstrated an effect of distance ($F_{19,722}=462.8$, $p<0.0001$), but failed to demonstrate effects of L-AAA treatment ($F_{2,722}=1.42$, $p=0.25$) or a distance x treatment interaction ($F_{(8,722)}=0.64$, $p=0.96$), on the number of intersections from the cell soma (**Figure 5.12**).

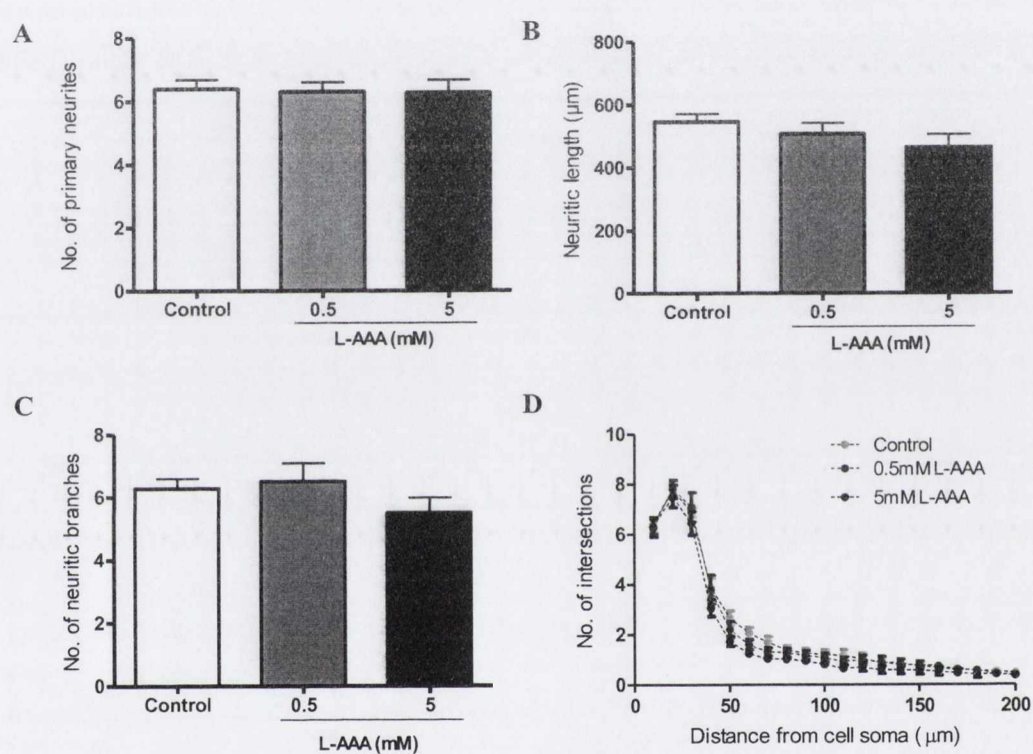


Figure 5.12: Effects of direct treatment of L-AAA on primary cortical neurons. Primary cortical neurons (DIV 3) were treated for 48 h with L-AAA (0.5, 5 mM) or control (NBM). Immunocytochemistry was performed at DIV 5. Neurons were identified using the neuronal structural protein β -III tubulin and the DAPI nuclear stain with fluorescence microscopy. Sholl analysis was performed on neurons to examine the number of primary neurites (**A**), the neuritic length (**B**), neuritic branches (**C**) and Sholl profile (**D**). Data are expressed as mean $\pm$ SEM, $n=13$ -15 coverslips per group representative of 3 independent experiments.

5.3.5.4 Effects of ketamine on astrocytic L-AAA CM-induced reduction in neurite outgrowth

Considering the reported neurotrophic effects of ketamine, we investigated whether ketamine could protect neurons from L-AAA CM induced atrophy. A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,79}=23.58$, $P<0.001$), but failed to demonstrate an effect of ketamine or a

L-AAA CM x ketamine interaction on the number of primary neurites. A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,74}=24.35$, $P<0.001$), and a L-AAA CM x ketamine interaction on the neuritic length ($F_{2,74}=5.19$, $P=0.008$). Finally, a two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,77}=13.5$, $P<0.001$) but failed to demonstrate an effect of ketamine or a L-AAA CM x ketamine interaction on the neuritic branches. *Post hoc* analysis revealed that neurons treated with L-AAA (0.5 and 5 mM) had fewer primary neurites and neuritic length ($P<0.01$) and neurons treated with L-AAA (5 mM) CM had fewer neuritic branches ($P<0.05$) when compared to control-treated neurons. Treatment with ketamine reversed the deficits in neuritic length as evidenced by an increase in neuritic length in neurons treated with ketamine and L-AAA (5 mM) CM compared to neurons treated with L-AAA (5 mM) CM alone ($P<0.05$) (Figure 5.13).

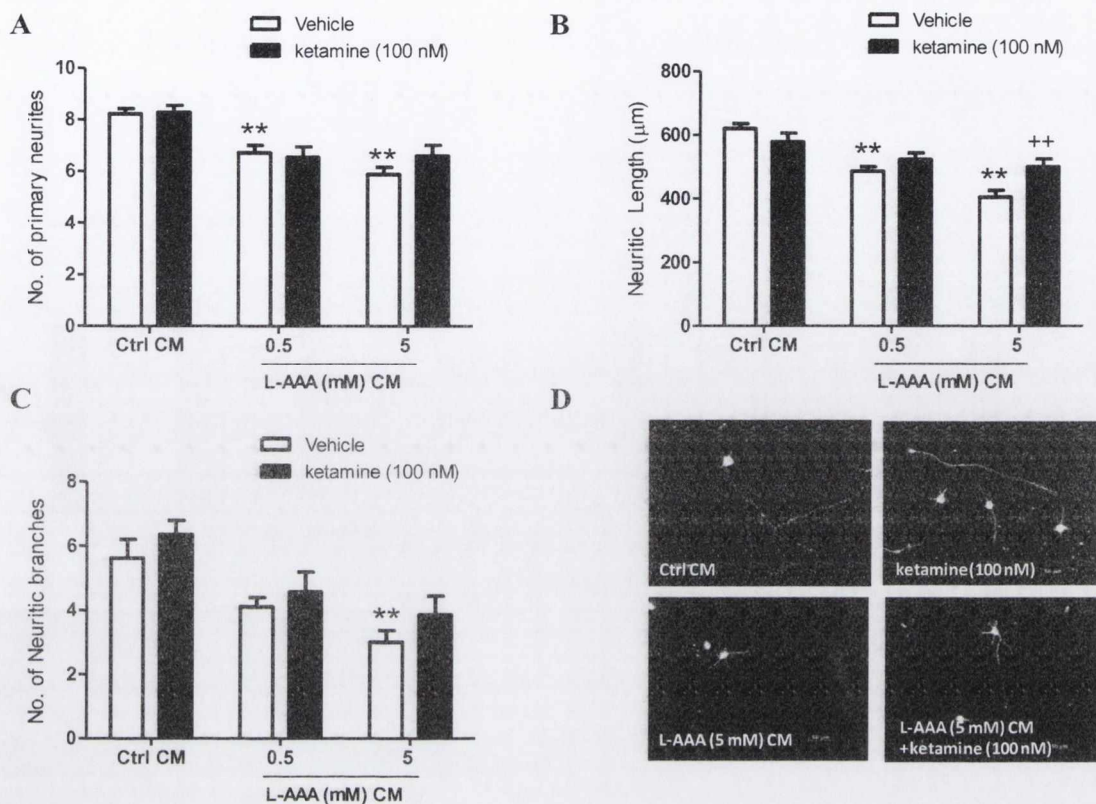


Figure 5.13: Effects of ketamine on astrocytic L-AAA CM-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of L-AAA CM (0.5 mM or 5 mM) and ketamine (1 μM). Sholl analysis was performed on neurons to examine (A) the number of primary neurites, (B) neuritic length and (C) the number of branches. Data are expressed as mean ± SEM, n=13-18 coverslips per group representative of 3 independent experiments. **P<0.01, *P<0.05 vs. ctrl CM, ++P<0.01 vs. respective vehicle (Student-Newman-Keuls). (D) Representative images of neurons identified using the neuronal structural protein β-III tubulin and the DAPI nuclear stain with fluorescence microscopy, Scale bar = 50 μm.

5.3.5.5 Effects of IC87201 on astrocytic L-AAA CM-induced reduction in neurite outgrowth

A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,84}=9.73$, $P<0.001$), but failed to demonstrate an effect of IC87201 or a L-AAA CM x IC87201 interaction on the number of primary neurites. A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,84}=8.94$, $P=0.003$), and an effect of IC87201 ($F_{1,84}=10.01$, $P=0.002$) but failed to demonstrate a L-AAA CM x IC87201 interaction on the neuritic length. Finally, a two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,84}=9.08$, $P<0.001$) but failed to demonstrate an effect of IC87201 or a L-AAA CM x IC87201 interaction on the neuritic branches. *Post hoc* analysis revealed that neurons treated with L-AAA (5 mM) CM had fewer primary neurites ($P<0.05$) and neurons treated with L-AAA (0.5 and 5 mM) CM had decreased neuritic length ($P<0.05$ and $P<0.01$ respectively) and fewer branches ($P<0.05$) when compared to control-treated neurons. Treatment with

IC87201 reversed the deficits in neuritic length as evidenced by an increase in neuritic length in neurons treated with IC87201 and L-AAA CM compared to neurons treated with L-AAA CM alone ($P<0.05$) (Figure 5.14).

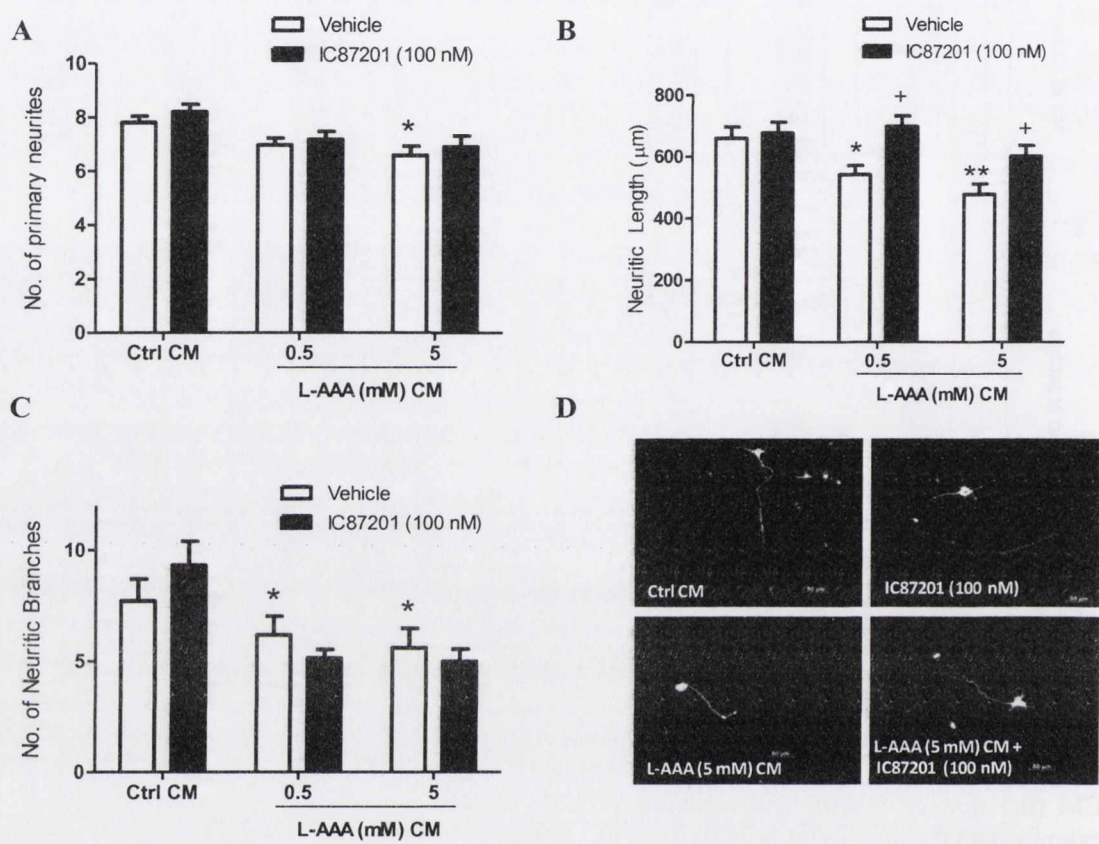


Figure 5.14: Effects of IC87201 on astrocytic L-AAA CM-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of L-AAA CM (0.5 mM or 5 mM) and IC87201(1 μM). Sholl analysis was performed on neurons to examine (A) the number of primary neurites, (B) neuritic length and (C) the number of branches. Data are expressed as mean ± SEM, n=14-17 coverslips per group representative of 3 independent experiments. ** $P<0.01$, * $P<0.05$ vs. ctrl CM, + $P<0.05$ vs. respective vehicle (Student-Newman-Keuls). (D) Representative images of neurons identified using the neuronal structural protein β-III tubulin and the DAPI nuclear stain with fluorescence microscopy, Scale bar = 50 μm.

5.3.5.6 Effects of TRIM on astrocytic L-AAA CM-induced reduction in neurite outgrowth

A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,60}=9.55$, $P<0.001$), an effect of TRIM ($F_{1,60}=11.66$, $P=0.001$), and a L-AAA CM x TRIM interaction ($F_{2,60}=9.78$, $P<0.001$) on the number of primary neurites. A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{2,56}=12.76$, $P<0.001$), an effect of TRIM ($F_{1,56}=25.83$, $P<0.001$), and failed to demonstrate a L-AAA CM x TRIM interaction on the neuritic length. Similarly, a two-way ANOVA demonstrated

an effect of L-AAA CM ($F_{2,61}=10.85$, $P<0.001$) and an effect of TRIM ($F_{1,61}=16.99$, $P<0.001$), but failed to demonstrate a L-AAA CM x TRIM interaction on the neuritic branches. *Post hoc* analysis revealed that L-AAA CM treated neurons had fewer primary neurites, decreased neuritic length and fewer branches ($P<0.01$) when compared to control-treated neurons. Treatment with TRIM was able to reverse these deficits, as evidenced by increased number of primary neurites ($P<0.01$), neuritic length ($P<0.05$) and branches ($P<0.01$) of neurons treated with L-AAA CM and TRIM when compared to neurons treated with L-AAA CM alone (**Figure 5.15**).

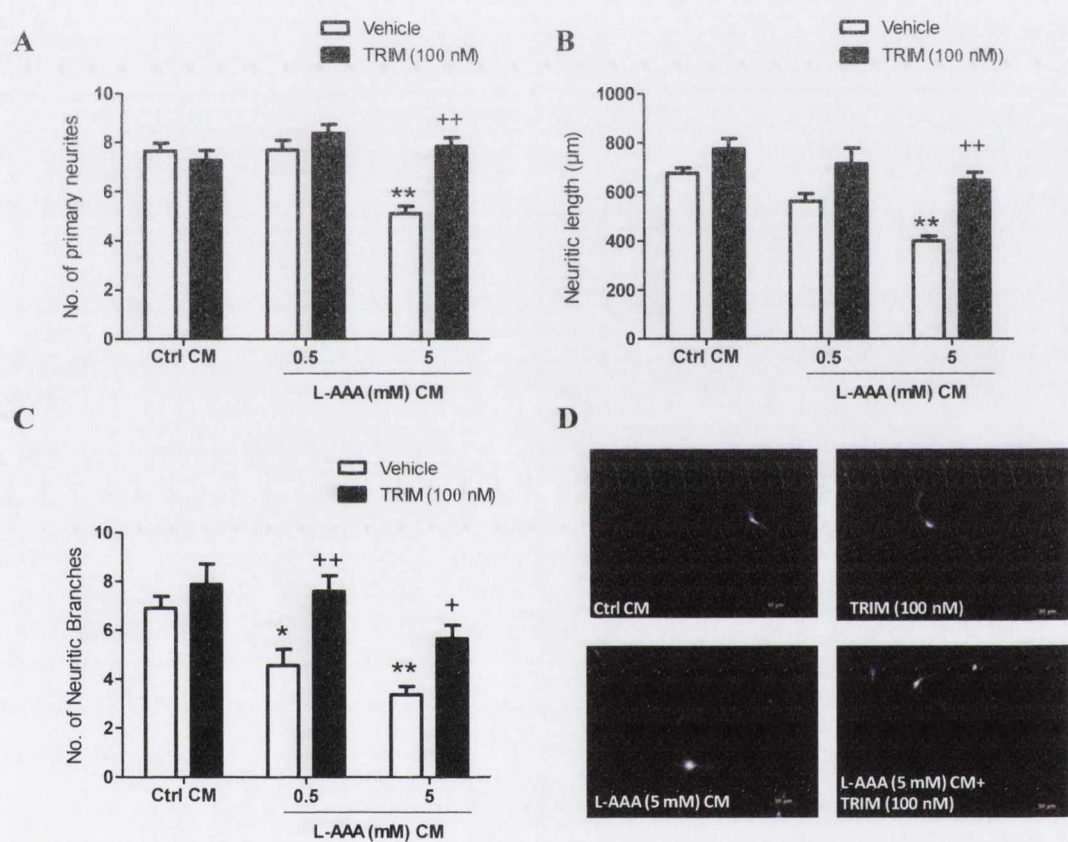


Figure 5.15: Effects of TRIM on L-AAA CM-induced reduction in neurite outgrowth. Primary cortical neurons (DIV 3) were treated for 24 h with a combination of L-AAA CM (0.5 mM or 5 mM) and TRIM (1 μM). Sholl analysis was performed on neurons to examine (A) the number of primary neurites, (B) neuritic length and (C) the number of branches. Data are expressed as mean ± SEM, n=12-14 coverslips per group representative of 3 independent experiments. ** $P<0.01$ vs. ctrl CM, ++ $P<0.01$ vs. respective vehicle (Student-Newman-Keuls). (D) Representative images of neurons identified using the neuronal structural protein β-III tubulin and the DAPI nuclear stain with fluorescence microscopy, Scale bar = 50 μm.

5.3.6 Effects of astrocyte CM on the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

Since astrocyte CM increased neurite outgrowth, we were interested in examining the effects of astrocyte CM on synapse number in primary cortical neurons (DIV 20) as assessed by the co-localisation of the pre- and post-synaptic markers, synaptophysin and PSD-95. One way ANOVA demonstrated effects of CM on the number of co-localised puncta ($F_{2,226} = 35.64$, $P < 0.001$). *Post hoc* analysis revealed that neurons treated with CM exhibited an increase in the number of co-localised puncta compared to control-treated neurons ($P < 0.001$). *Post hoc* analysis also demonstrated that neurons treated with L-AAA (0.05 and 0.5 mM) CM showed a decrease in the number of co-localised puncta when compared to neurons treated with CM alone and also when compared to control-treated neurons ($P < 0.001$) (Figure 3.16).

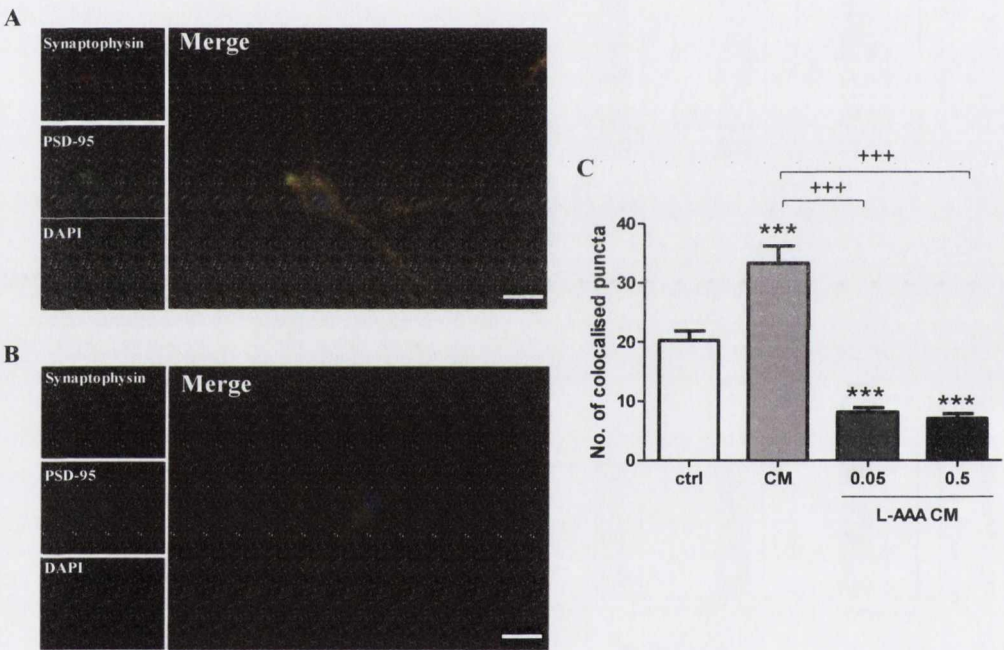


Figure 5.16: Effects of astrocyte CM on the co-localised expression of synaptophysin and PSD-95 in synaptic puncta. Neurons were identified using the pre-synaptic marker synaptophysin (red), the post-synaptic marker PSD-95 (green), and the DAPI nuclear stain (blue) with fluorescence microscopy, (A) Representative image of primary cortical neurons (DIV 21) treated with astrocyte conditioned media (CM) or (B) L-AAA (0.5 mM) CM. Scale Bar = 20 μm. Quantification of co-localised synaptic proteins was conducted using ImageJ software. (C) Neurons were treated for 24 h with normal culture media (ctrl), conditioned media (CM) derived from healthy astrocytes, or CM derived from astrocytes treated with L-AAA (0.05 or 0.5 mM) for 24 h (L-AAA CM). Data are expressed as mean ± SEM, n=50-80 neurons per group representative of 3 independent experiments. *** $P < 0.001$ vs. ctrl, +++ $P < 0.001$ L-AAA CM vs. CM (Student-Newman-Keuls).

5.3.7 Effects of NMDA-R/NO signalling inhibition on astrocytic L-AAA CM-induced reduction in the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

5.3.7.1 Effects of Ketamine on astrocytic L-AAA CM-induced reduction in the co-localised expression of synaptic proteins

A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{1,109}=19.16$, $P<0.01$), an effect of ketamine ($F_{1,109}=29.85$, $P<0.01$) and a L-AAA CM x ketamine interaction ($F_{1,109}=9.33$, $P<0.01$) on the number of co-localised puncta. *Post hoc* analysis revealed that L-AAA CM-treated neurons had fewer co-localised puncta when compared to control-treated neurons ($P<0.01$). Treatment with ketamine attenuated this reduction as evidenced by an increase in co-localised puncta ($P<0.01$) in neurons treated with L-AAA CM and ketamine when compared to L-AAA CM-treated neurons alone ($P<0.01$) (**Figure 3.17A**).

5.3.7.2 Effects of TRIM on astrocytic L-AAA CM-induced reduction in the co-localised expression of synaptic proteins

A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{1,91}=19.98$, $P<0.01$), an effect of TRIM ($F_{1,91}=39.5$, $P<0.01$) and a L-AAA CM x TRIM interaction ($F_{1,91}=15.69$, $P<0.01$) on the number of co-localised puncta. *Post hoc* analysis revealed that L-AAA CM-treated neurons had fewer co-localised puncta when compared to control-treated neurons ($P<0.01$). Treatment with TRIM attenuated this reduction as evidenced by an increase in co-localised puncta ($P<0.01$) in neurons treated with L-AAA CM and TRIM when compared to L-AAA CM-treated neurons alone ($P<0.01$) (**Figure 3.17B**).

5.3.7.3 Effects of IC87201 on astrocytic L-AAA CM-induced reduction in the co-localised expression of synaptic proteins

A two-way ANOVA demonstrated an effect of L-AAA CM ($F_{1,97}=5.07$, $P<0.01$), an effect of IC87201 ($F_{1,97}=33.45$, $P<0.01$) and a L-AAA CM x IC87201 interaction ($F_{1,97}=47.89$, $P<0.01$) on the number of co-localised puncta. *Post hoc* analysis revealed that L-AAA CM-treated neurons had fewer co-localised puncta when compared to control-treated neurons ($P<0.01$). Similar to the effects observed with ketamine and TRIM, treatment with IC87201 also attenuated L-AAA CM induced reductions in co-localised puncta as demonstrated by an increase in co-localised puncta ($P<0.01$) in neurons treated with L-AAA CM and IC87201 when compared to L-AAA CM-treated neurons alone ($P<0.01$) (**Figure 3.17C**).

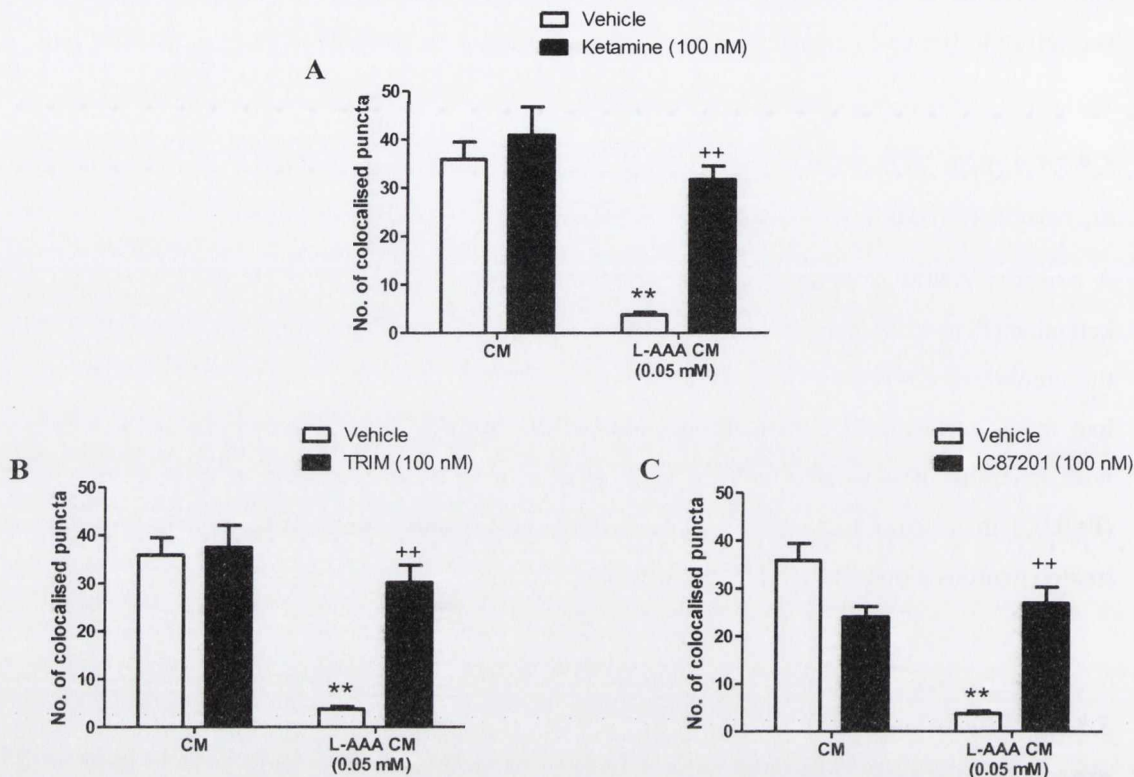


Figure 5.17: Effects of NMDA-R/NO signalling inhibition on astrocytic L-AAA CM-induced reduction in the co-localised expression of synaptophysin and PSD-95. Quantification of the co-localised expression of synaptophysin and PSD-95 in synaptic puncta was conducted using ImageJ software. Neurons (DIV 20) were treated for 24 h with a combination of L-AAA (0.05 mM) in addition to (A) ketamine, (B) TRIM or (C) IC87201 for 24 h before fixation and immunocytochemistry for analysis of the number of co-localised puncta. Data are expressed as mean $\pm$ SEM, $n=25-30$ neurons per group representative of 3 independent experiments. ** $P<0.01$ vs. ctrl CM, ++ $P<0.001$ vs. respective vehicle (Student-Newman-Keuls).

5.4 Discussion

The results of the present investigation demonstrate that L-AAA induces a selective astrocyte toxicity *in vitro* which involves changes in the expression of the glutamate transporters, GLAST and GLT-1, and leads to alterations in mitochondrial metabolism. We employed the use of an *in vitro* model consisting of astrocyte CM to investigate the effects of soluble astrocyte-derived factors in neurite outgrowth, as well as on the number of co-localised synaptic protein puncta as a measure of synapse number. Our results demonstrate that CM derived from healthy astrocytes increases neurite growth in neurons (DIV 3) and leads to a robust increase in synapse number in more mature neuronal cultures (DIV 20). Following on from this, we show that exposing astrocytes to L-AAA leads to the production of CM which reverses the trophic effects of healthy astrocyte CM on neurite outgrowth and reduces the co-localised expression of synaptic proteins, indicating a reduction in synapse number. We show that the atrophic nature of L-AAA CM on neurons involves glutamatergic stimulation by demonstrating that the co-treatment of neurons with the NMDA-R antagonist ketamine, as well as nNOS inhibitor TRIM and small molecule inhibitor of the PSD-95/nNOS interaction, IC87201, can protect against L-AAA CM-induced effects on neurite outgrowth and synapse number. These results highlight the importance of healthy astrocytes, and the implications of astrocytic dysfunction, in the processes governing neuronal and synapse formation. Moreover, this study supports the use of L-AAA as a tool to investigate astrocytic dysfunction and provides promising insights into glutamatergic modulation as a potential strategy for neuronal remodelling in CNS disorders associated with astroglial dysfunction and neuronal atrophy.

5.4.1 L-AAA selectively reduces the viability of cultured astrocytes

The results demonstrate that L-AAA (5 mM) induces a reduction in mixed glial viability after 48 h. Since mixed glial cultures are composed of both astrocytes and microglia, we isolated cultures of both of these cell types and showed that L-AAA (5 mM) reduces purified astrocytic viability after 48 h but not purified microglial viability, suggesting that L-AAA selectively impairs astrocytic function while leaving microglia unaffected. These results are in agreement with data from the literature which demonstrated reduced viability at similar concentrations (1 – 10 mM) in mixed glial and astrocytic cultures as analysed by MTS assay (Brown and Kretschmar, 1998). Interestingly however, these authors provided evidence to suggest that co-culture with microglia is capable of reducing the toxicity of L-AAA in mixed glial cultures. This effect was attributed to glutamate release by microglia - which presumably reduces toxicity by competing with L-AAA for uptake into astrocytes (McBean, 1994). Our results are in contrast to these reports as we demonstrate comparable levels of toxicity between astrocytes and mixed glial cultures and indicate that co-culture of microglia with astrocytes has no effect on L-AAA-induced toxicity. These differences could be attributed to differences in experimental designs, including the level of

maturation of cells at the time of treatment (DIV 13 vs. day of seeding), treatment duration (48 h vs. 5 days) and the type of astrocytes under investigation (mixed vs. type 1/ type 2) (Brown and Kretschmar, 1998). We also demonstrate that L-AAA (5 mM) induces a sustained reduction in astrocyte viability *in vitro* that cannot be reversed by the removal of L-AAA treatment and replacement with fresh culture media. *In vivo* studies have indicated that injection of L-AAA (50 ug/ ul) into the PFC of rats induces a transient astroglial dysfunction within 7-24 h, with complete recovery after 48 h as assessed by an increase in GFAP positive astroglial cells at the lesion site (Banar and Duman, 2008). These results highlight differences between astrocytic responses to L-AAA *in vitro* and *in vivo* and indicate the ability of astrocytes to proliferate and recover from L-AAA administration *in vivo*.

Early research provided evidence to suggest that L-AAA can exhibit excitotoxic effects on neurons at high concentrations *in vitro* (MacDonald and Wojtowicz, 1982). Here, we show that L-AAA does not affect the viability of purified neuronal cultures at DIV 3-4. It was of importance to establish that L-AAA causes no overt reductions in neuronal viability in our model since we were mainly interested in examining the impact of astrocytic dysfunction and astrocyte secreted factors on neuronal responses, rather than the direct effect of L-AAA *per se*. This is also of importance with respect to animal studies using L-AAA, in order to establish that the behavioural consequences observed following astrocytic ablation are not due to off-target effects of L-AAA on neurons. In this regard, previous studies have shown that L-AAA does not cause neurotoxicity and acts specifically on astrocytes *in vivo* (Khurgel et al., 1996) In addition, the depressive-like behavioural effects observed following L-AAA microinjection into the rat PFC have been shown to be attributable to astrocytic ablation since injection of the selective neurotoxin, ibotenate, to the same region did not result in a similar behavioural phenotype (Banar and Duman, 2008).

Since L-AAA is a glutamate analogue and differs from glutamate by a single methylene group, it was important to establish whether glutamate itself resulted in a similar reduction in astrocyte viability as observed with L-AAA. Previous research has found that treatment of astrocyte cultures to glutamate (1 mM) for 24 h leads to increased LDH release in mixed glial and astrocyte cultures, and that this toxicity is mediated through oxidative stress mechanisms (Chen et al., 2000). Our results are in contrast to these findings however, and we show that glutamate treatment for 24 h or 48 h does not decrease viability of purified astrocytes at any concentration tested (0.05 - 5 mM). Instead, our findings demonstrate that glutamate treatment (0.05 and 0.5 mM) increases the viability of astrocytes after 24 h while the highest concentration (5 mM) had no effects on viability. These effects were transitory and were not sustained after 48 h. Since the Alamar blue viability assay used in this experiment is based on metabolic mitochondrial activity of cells (O'Brien et al., 2000), these results could suggest that glutamate stimulation leads to a transient increase in metabolic activity after 24 h. Importantly, these results indicate that the mode

of toxicity associated with the application of L-AAA to astrocytes does not generalise to its analogue glutamate and further validates the use of L-AAA as an astrocyte specific toxin.

5.4.2 L-AAA affects the expression of glial-specific markers and astrocytic mitochondrial respiration

We further characterised the mechanism of action of L-AAA by assessing its effect on mRNA expression patterns of glial specific markers as well as general markers of inflammation and apoptosis. Our research demonstrates that the treatment of primary cortical astrocytes (DIV 13) with L-AAA for 24 h (0.5 mM and 5 mM) decreases mRNA expression levels of the prototypical astrocyte marker, GFAP. These results suggest L-AAA alters astrocytic morphology and cytoarchitecture at a subtoxic concentration (0.5 mM) and time point (24 h). Alterations in GFAP expression are a hallmark of dysfunctional astrocytes, and increases in GFAP expression and immunoreactivity are widely considered a reliable marker for reactive astrocytes (Sofroniew and Vinters, 2010). Our results, which demonstrate a decrease in GFAP expression, suggest that L-AAA does not elicit the classical ‘reactive astrogliosis’ response in astrocytes (Sofroniew, 2009, Zamanian et al., 2012), but instead leads to a functional impairment relating to reduced expression of this cell marker. In terms of L-AAA treatment *in vivo*, research has demonstrated a reduction in the density of GFAP-positive cells around the lesion site following L-AAA injection (Banar and Duman, 2008, Wang and Hatton, 2009), with these effects most likely attributable to a reduction in the number of astrocytes present in the area. In addition, prior research has found that astrocytic ablation with L-AAA can result in a positive effect on disease pathology, by using L-AAA as a strategy to prevent the detrimental effects associated with reactive astrocytes in animal models of epilepsy (Zhu et al., 2012).

Our data demonstrate a robust increase in S100B mRNA expression following 24 h treatment with L-AAA (5 mM). S100B is an astrocyte-derived calcium binding protein which is known to possess neurotrophic roles in normal physiological conditions, but can be secreted in large amounts and contribute to neurotoxicity under pathological conditions (Hu et al., 1997, Donato and Heizmann, 2010). Overexpression and elevated levels of this astrocytic marker have been documented in several pathological states, most notably Alzheimer’s disease - in which it has been linked to neuronal atrophy (Mrak and Griffin, 2001) - but also in stroke (Zhao and Rempe, 2010) and major depression (Schroeter et al., 2002). The results presented here demonstrate that L-AAA results in a robust increase in S100B expression, which may have consequences for the detrimental knock-on effects of dysfunctional L-AAA treated astrocytes on neuronal growth.

We also examined the effects of L-AAA on the mRNA expression patterns of the astrocyte specific glutamate transporters, GLT1 and GLAST. We demonstrate that L-AAA (5 mM) leads to an increase in GLT- 1 mRNA expression, while L-AAA at both 0.5 mM and 5 mM decrease

GLAST mRNA expression compared to control-treated astrocytes. These results lend support to data in the literature which indicate that L-AAA induced astrocyte toxicity involves alterations in glutamate transporter systems (McBean, 1994, Tsai et al., 1996, Brown and Kretzschmar, 1998). Although we cannot determine glutamate transporter activity levels or function from the current data, the alterations in mRNA expression of these transporters suggest that they may be dysfunctional, which may lead to a variety of cellular consequences including potential glutamate efflux from astrocytes - a phenomenon known to occur in pathological conditions such as ischemia and excitotoxicity (Malarkey and Parpura, 2009, Hamilton and Attwell, 2010).

The results of the present investigation demonstrate that L-AAA does not induce the expression of the inflammatory marker, iNOS or the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF α). These data suggest that the mode of toxicity of L-AAA is not related to the induction of an inflammatory phenotype in astrocytes. This was of interest to rule out, as various types of insults have been shown to induce expression of iNOS in astrocytes and contribute to neuronal toxicity through the generation of excess NO (Bal-Price and Brown, 2001, Brown and Bal-Price, 2003, Sofroniew, 2005). In addition, astrocytic release of TNF α is upregulated in response to a range of stimuli including IFN-gamma, IL-6 and LPS (Chung and Benveniste, 1990). Since our data indicate L-AAA (5 mM) reduces astrocyte viability, we assessed whether L-AAA induced the expression of caspase-3, a major executioner caspase associated with apoptosis (Elmore, 2007). Previous research has suggested that astrocytes die after prolonged exposure to L-AAA (1 mM), suggesting the activation of delayed apoptotic mechanisms (Nishimura et al., 2000). We demonstrate that L-AAA has no effect on caspase-3 mRNA expression after 24 h of treatment, which suggests that L-AAA-dependent cell toxicity does not activate this marker of apoptosis. Although caspase activity or TUNEL staining would provide a more thorough measure of apoptosis, increases in caspase-3 mRNA expression is widely reported to occur after many apoptotic stimuli and is considered a reliable marker of apoptotic cells (Harrison et al., 2000). Finally, we show that L-AAA does not affect the expression of the enzyme responsible for its metabolism, kynurenine aminotransferase II (KATII). This enzyme is also responsible for catalysis of kynurenine to the neuroprotective excitatory amino acid receptor antagonist kynurenic acid. Prior studies have shown that L-AAA inhibits astrocytic kynurenic acid synthesis *in vitro* and *in vivo* in the rat hippocampus, as well as decreasing KATII activity (Wu et al., 1995, Chang et al., 1997). We also demonstrate that astrocytes treated with L-AAA (0.5 and 5 mM) for 24 h exhibit reductions in oxygen consumption rate (OCR), an indicator of mitochondrial respiration (Ferrick et al., 2008). Inhibition of cellular respiration in astrocytes is well known to occur in response to many stimuli, most prominently by NO which reversibly binds and inhibits cytochrome oxidase (Hurst et al., 1996, Brown and Borutaite, 2002), an effect which has been shown to contribute to neurodegeneration in neuron-astrocyte co-cultures (Bal-Price and Brown,

2001, Brown and Bal-Price, 2003). Together, these results suggest that L-AAA-induced astrocyte dysfunction involves specific alterations in glutamate transport systems and mitochondrial respiration. The results presented here do not support the hypothesis that L-AAA-mediated astrocytic dysfunction involves the induction of a pro-apoptotic state or inflammatory phenotype.

5.4.3 L-AAA-induced astrocytic dysfunction leads to neuronal atrophy

The data presented in this investigation firstly demonstrate that CM derived from healthy astrocytes increases neurite outgrowth of primary cortical neurons (DIV 3). Specifically, we show that when CM is harvested from healthy astrocytes after 24 h and then transferred to neurons for 24 h, an increase in neurite outgrowth is observed in terms of the number of primary neurites and the number of neuritic branches at 20 - 50 μm from the cell soma. When CM is harvested from astrocytes after 48 h, the neurite outgrowth inducing effect is enhanced even further, with the resulting CM capable of increasing all parameters of neurite outgrowth tested, including the number of primary neurites, the neuritic length and the number of neuritic branches at 10 - 20 μm from the cell soma. These results demonstrate that unstimulated astrocytes produce a trophic media which can increase neurite outgrowth *in vitro*. Such findings are in line with prior research indicating the neuroprotective effect of astrocyte CM against various noxious stimuli *in vitro*. For instance, pre-treatment of rat spinal cord neurons with unstimulated astrocyte CM is capable of attenuating glutamate-induced apoptotic cell death (Lu et al., 2015). CM from astrocytes has also recently shown neuroprotective effects against the environmental chemical methylmercury, an effect attributed to the release of BDNF and NGF (Takemoto et al., 2015). Furthermore, astrocyte CM protects primary cultures of hippocampal neurons from corticosterone induced damage as measured by TUNEL labelling (Zhu et al., 2006), as well as partially attenuating against excess reactive oxygen and nitrogen species (Tanaka et al., 1999). Such results validate the use of astrocyte CM as a method of enhancing neurotrophic support for neurons and providing protection against harmful stimulants.

Following on from this, we investigated the effects of transferring the CM from L-AAA treated astrocytes (L-AAA CM) onto neurons to assess whether dysfunctional astrocytes affect neurite outgrowth. The data show that CM derived from astrocytes treated with L-AAA (5 mM) for 24 h reduces the neuritic length and the number of neuritic branches of neurons compared to neurons treated with CM from untreated astrocytes. In addition, the treatment of neurons with CM derived from astrocytes treated for 48 h with L-AAA exacerbates the reduction in outgrowth - which may be in line with the reduction in viability observed at this time point – and leads to a reduction in the all parameters of neurite outgrowth under assessment after 24 h of neuronal treatment. These results show that, although L-AAA does not result in overt reductions in viability to astrocytes after 24 h, the CM from these astrocytes causes a significant decrease in neurite length and branching. Reductions in outgrowth are also observed with the CM obtained from a lower

concentration of L-AAA (0.5 mM) than is required for reductions in viability. These results demonstrate that L-AAA abolishes the trophic effects of astrocyte CM on neurite outgrowth. Importantly, L-AAA was also applied directly to neurons, with the results showing that L-AAA does not decrease neurite outgrowth directly, but instead acts on astrocytes, altering their function and impairing their ability to promote neurite growth.

These results are of interest in the context of research indicating that an astrocyte dysfunction may contribute to neuronal atrophy *in vivo*. For example, recent studies have demonstrated that L-AAA injection into the medial PFC of rats caused an astrocyte ablation accompanied by progressive neuronal loss and atrophy of surviving neurons in the lesion vicinity (Lima et al., 2014). In addition, astrocytic dysfunction represents one of the earliest responses after ischemic stroke and precedes neuronal cell death in the core (Liu et al., 1999, Panickar and Norenberg, 2005), indicating that the loss of astrocytes contributes to neuronal atrophy in ischemia. This may occur through the loss of essential metabolic, structural or trophic support provided by astrocytes to neurons or through astrocytic upregulation of inflammatory or neurotoxic mediators.

5.4.4 L-AAA abolishes the synaptogenic effects of astrocyte CM

The data presented here show that treatment (24 h) of primary cortical neurons (DIV 20) with astrocyte CM results in a robust increase (~50%) in the number of co-localised synaptophysin and PSD-95 puncta. In contrast, the data demonstrates a reduction in the co-localised puncta after treatment with L-AAA (0.05 and 0.5 mM) CM compared to neurons treated with both CM from untreated astrocytes as well as neurons treated with control media. Immunocytochemistry assays based on the quantification of the co-localised expression of specific pre- and post-synaptic proteins have been used by many other research groups as a reliable method for the quantification of synapses (Ippolito and Eroglu, 2010, Roqué et al., 2014). Our results, which show increases in co-localised synaptophysin and PSD-95 staining, therefore suggest that astrocyte CM leads to a robust increase in synapse number in primary cortical neurons *in vitro*, which is in agreement with literature indicating a role for astrocyte-derived factors in synapse formation (Pfrieger and Barres, 1997, Ippolito and Eroglu, 2010). Furthermore, our results indicate that dysfunctional, L-AAA treated astrocytes abolish the synaptogenic effects of astrocyte CM on synapse formation. Such observations highlight the importance of astrocyte-derived factors in synapse formation and development, and may implicate dysfunctional astrocytes as a potential contributing factor in synapse loss associated with many CNS disorders including schizophrenia (Harrison, 2004) major depressive disorder (MDD) (Cotter et al., 2001, Rajkowska and Miguel-Hidalgo, 2007) and Alzheimer's disease (Shankar et al., 2007).

5.4.5 Protection against L-AAA CM-induced neuronal atrophy by NMDA-R/NO signalling inhibition

Since our previous investigations found that NMDA-R antagonists and inhibitors of the PSD-95/nNOS interaction possess the ability to counteract NMDA-induced neurite regression [Chapter 3 and Doucet et al. (2015)], we sought to investigate whether these compounds could also provide protection against L-AAA CM-induced deficits in neurite outgrowth and synapse number. We now demonstrate that the NMDA-R antagonist ketamine (100 nM) can prevent L-AAA (5 mM) CM-induced decreases in the neuritic length of cortical neurons. A similar protective effect was observed with the small molecule inhibitor of the PSD-95/nNOS interaction, IC87201 (100 nM), which counteracted L-AAA (0.5 and 5 mM) CM-induced decreases in neuritic length. In addition, the nNOS inhibitor TRIM (100 nM) was capable of attenuating L-AAA (5 mM) CM-induced decreases in the number of primary neurites and neuritic length as well as protecting against L-AAA (0.5 and 5 mM) CM-induced decreases in the number of neuritic branches. In terms of synapse number, we demonstrate that ketamine, TRIM and IC87201 (100 nM) provide protection against L-AAA (0.05 mM) CM-induced reductions in the co-localised expression of synaptophysin and PSD-95. These results demonstrate that modulation of the NMDA-R/PSD-95/nNOS signalling pathway in neurons can protect against atrophy and potential synapse loss associated with L-AAA-induced astrocytic dysfunction. These results may provide insights into the potential of NMDA-R antagonists and downstream inhibitors of the PSD-95/nNOS interface as a strategy for attenuating neuronal growth impairments related to dysfunctional astrocytes. In this regard, L-AAA microinjection in the PFC leads to a similar glial pathology and depressive-like behaviours in rats as those observed following chronic unpredictable stress (CUS) paradigms, and the glutamate modulating drug riluzole has been found to reverse both CUS-induced glial dysfunction and depressive-like behaviours (Banasr et al., 2010). Subanaesthetic doses of ketamine have displayed promising neurotrophic effects *in vitro* by enlarging growth cones in cortical neurons (Brown et al., 2015) and increasing neurite outgrowth in neuronal cell lines (Robson et al., 2012). Furthermore, ketamine's antidepressant-like effects are widely reported to involve an increase in synaptogenesis and spine formation in the PFC in animal models of depression (Duman and Li, 2012), further supporting the neurotrophic potential of NMDA-R antagonism.

5.4.6 Conclusions

In conclusion, the results of the current investigation firstly demonstrate that L-AAA exerts a selective toxicity in astrocytes which involves alterations in the expression of glutamate transporters and a reduction in mitochondrial respiration. Reductions in viability are only evident after prolonged (48 h) exposure to a high concentration (5 mM) of L-AAA. These results help to validate the use of L-AAA as a tool for selective astrocytic ablation in cell culture and animal

models. We were particularly interested in the effects of secreted factors produced by dysfunctional astrocytes on neurite outgrowth and synapse number. We firstly demonstrated that CM from healthy astrocytes increases neurite outgrowth and synapse formation while CM from L-AAA treated astrocytes (L-AAA CM) reduces both of these measures of neuronal complexity. These results demonstrate that dysfunctional astrocytes can profoundly increase the susceptibility of neurons to atrophy, and highlights the potential for targeting NMDA-R/NO neuronal signalling as a strategy to overcome atrophic effects associated with astrocytic dysfunctions in disorders involving altered glutamate transmission.

Chapter 6: General Discussion

6.1 Summary of main findings

Neuronal atrophy and structural alterations in dendrites and synapses represent a common pathological feature of brain injury and disease (Fiala et al., 2002, Kulkarni and Firestein, 2012). The current research used an *in vitro* model composed of primary cortical neurons to investigate the effects of glutamate and modulation of specific protein interactions in the NMDA-R/NO signalling pathway on neurite outgrowth and synaptic protein co-localisation as a measure of synapse number. The main findings of this research demonstrated that excessive glutamatergic stimulation induced neurite atrophy and resulted in a reduction in the co-localised expression of pre-synaptic synaptophysin and post-synaptic PSD-95 in synaptic puncta. These effects were mediated through the NMDA-R, and were prevented by NMDA-R antagonism and by small molecular weight inhibitors of the PSD-95/nNOS interaction as well as through peptide-mediated inhibition of the NOS1AP/nNOS interaction. A role for NO was demonstrated in neurite outgrowth, with the results demonstrating that pharmacological inhibition of nNOS and of the main NO receptor, sGC, can protect against reductions in neurite outgrowth induced by the NO donor, SNP, or the NO precursor, L-arginine. The results also demonstrated a distinct role for stress activated protein kinases in the reductions in neurite outgrowth induced by NMDA-R activation. In particular, our findings demonstrate the ability of p38 MAPK inhibition to attenuate NMDA-induced reductions in outgrowth and synaptic protein co-localisation. Investigating the effects of astrocyte-derived factors in the modulation of neuronal morphology was another main focus of this thesis. We demonstrated that factors derived from healthy astrocytes increased neurite outgrowth and resulted in an increase in the co-localised expression of synaptic markers. Conversely, induction of astrocytic ablation through the use of the toxin L-AAA abolished these trophic effects. Overall, these findings identify a pathway downstream of NMDA-R activation which plays a role in neurite outgrowth responses, and demonstrates that targeting specific elements of this pathway protects against neuronal atrophy induced by NMDA and promotes neurotrophic effects. The results of this research also demonstrate an important role for astrocyte-derived factors in neurite outgrowth and synapse morphology and highlight the detrimental effects of astrocyte dysfunction on neuronal structure.

6.2 NMDA-R/NO/cGMP signalling: A pathway to neuronal atrophy

The earliest signs of glutamate excitotoxicity involve neuronal structural modifications such as dendritic beading and spine loss which precede cell death (Monnerie et al., 2003). Interestingly, these structural modifications are known to be reversible, and neurons are capable of recovering from early glutamate-induced damage (Ikegaya et al., 2001, Greenwood and Connolly, 2007). Targeting these early events therefore, may provide a means to prevent subsequent cell death, restore neuronal connectivity and promote functional recovery in many disease states. Although the detrimental effects of excess glutamate and NMDA which lead to neuronal death are well described (Mehta et al., 2013), the structural changes associated with early/subtoxic NMDA exposure are not as well understood. In this thesis we demonstrated that lower concentrations of glutamate increased neurite outgrowth and showed that this effect was blocked by the AMPA-R antagonist, NBQX, thereby indicating a role for AMPA-R in mediating the neurotrophic effects of glutamate. In contrast, our results show that higher concentrations of glutamate and NMDA in addition to glycine (a co-agonist of the NMDA-R) reduced neurite outgrowth and decreased the co-localised expression of synaptic proteins. These results demonstrate a biphasic effect of glutamate on neurite outgrowth responses, and this is in agreement with data from the literature suggesting a requirement for AMPA-R activation in the maintenance of dendritic spines (McKinney et al., 1999) and in glutamate-mediated dendritic growth in motoneurons (Metzger et al., 1998).

Although the processes of neuronal growth and synapse formation are distinct, it is known that the mechanisms underlying both processes converge on pathways involved in cytoskeletal regulation and actin dynamics (Hering and Sheng, 2001, Luo, 2002, Ethell and Pasquale, 2005) and it is therefore of interest to discuss the findings presented in this thesis in relation to potential mechanisms that link NMDA-R activation to proteins directly involved in cytoskeletal alterations (**Figure 6.1**). In this regard, NMDA has previously been shown to rapidly dephosphorylate microtubule associated protein 2 (MAP2) in hippocampal neurons, an effect suggested to occur via activation of the Ca^{2+} /CaM-dependent protein phosphatase, calcineurin (Halpain and Greengard, 1990, Buddle et al., 2003). MAP2 is an important cytoskeletal protein involved in microtubule stabilisation, organelle transport and anchorage of regulatory kinases to the cytoskeleton (Sanchez et al., 2000), and its dephosphorylation by NMDA may therefore contribute towards the observed neurite atrophy and potential synapse loss.

The NMDA-R is functionally coupled to nNOS via the PSD-95 (Christopherson et al., 1999), and subsequent production of NO by nNOS leads to the activation of the main NO receptor, sGC, which catalyses the conversion of GTP to cGMP. Our results implicate a role for the NMDA-R/NO/cGMP pathway in neurite outgrowth responses as we demonstrated that inhibition of nNOS

and sGC was capable of restoring neurite outgrowth to basal levels following stimulation with NMDA or the NO donor, L-arginine. PSD-95, NO and sGC activity have previously been shown to impact upon synapse development and neurite outgrowth responses (Hindley et al., 1997, El-Husseini et al., 2000, Charych et al., 2006, Shelly et al., 2010, Tseng and Firestein, 2011) and NO is known to directly regulate growth cone dynamics and filopodial extension (Bicker, 2005). In addition, NO has been reported to cause growth cone collapse in dentate gyrus cells through a cGMP-dependent pathway (Yamada et al., 2006). Another report demonstrated that NO mediates synaptic retraction and elimination in motoneurons through the activation of a GTPase involved in cytoskeletal dynamics, RhoA/Rho kinase (ROCK) signalling, via cGMP/PKG pathway activation (Sunico et al., 2010). These results may be of interest with regard to the research presented in this thesis, as they directly link nNOS-dependent NO formation to cytoskeletal changes via cGC, and could therefore represent a potential mechanism to explain changes in neurite outgrowth and synaptic protein co-localisations in response to NMDA and NO. sGC is expressed both pre- and post- synaptically and NO may therefore exert retrograde effects in the pre-synaptic neuron through these mechanisms.

Another potential link between NMDA- and NO-mediated decreases in neurite outgrowth and synapse loss may involve inhibition of BDNF secretion which is well known to be an important regulator of neuronal growth, axon arborisation and synaptogenesis, and is an important functional regulator at glutamatergic synapses (Minichiello, 2009, Sunico et al., 2010). Interestingly, activation of the NO/cGMP pathway results in downregulation of BDNF through a PKG-dependent mechanism in cultured hippocampal neurons (Canossa et al., 2002) and NO-mediated growth cone collapse and axon retraction in retinal axons can be prevented by pre-treatment with BDNF (Ernst et al., 2000), indicating that NO may lead to axon retraction through decreases in this neurotrophin. NO also acts independently of sGC activation and is known to exert cellular effects via direct S-nitrosylation of target proteins, a process which is increasingly implicated in synaptic plasticity and neurogenesis (Hardingham et al., 2013, Santos et al., 2014). It is possible that NO may contribute to neuronal regression and synapse loss through S-nitrosylation of proteins involved in cytoskeletal dynamics. For instance, prior studies observed growth cone collapse and axonal actin reconfiguration in response to NO-mediated S-nitrosylation of microtubule associated protein 1B (Stroissnigg et al., 2007).

Our work also establishes a role for stress activated protein kinases, p38 MAPK and JNK, in the reduction of co-localised synaptic markers, and a distinct role for p38 activation in modulating neurite retraction following exposure to NMDA. Previous research has demonstrated that NMDA-R-mediated p38 activation requires the PSD-95/nNOS interaction (Cao et al., 2005, Soriano et al.,

2008) and more recent research identified that excitotoxic stimulation of cultured neurons recruits the p38 activator, MKK3, via activation of NOS1AP (Li et al., 2013). Here, we show that a peptide inhibitor of the NOS1AP/nNOS pathway, as well as direct inhibitors of p38 MAPK can attenuate NMDA-induced reductions in neurite growth and co-localised synaptic markers. Activation of p38 results in diverse cellular responses through the phosphorylation and activation of a wide range of substrates, and p38 activation is well known to play a role in mediating apoptosis and cell death under stressful or inflammatory stimuli (Johnson and Lapadat, 2002). In terms of the role of p38 in neurite outgrowth responses, it has been shown that activated p38 phosphorylates several cellular targets that may impact upon the cytoskeleton and cause a reorganisation of neuronal structural proteins. For instance, p38 is known to activate microtubule-associated protein Tau and lead to destabilisation of microtubules (Corrêa and Eales, 2012). In addition, two well known substrates for p38 - MAPK activated protein kinases 2 and 3 (MK-2 and -3) - are also known to play a prominent role in actin reorganisation (Roux and Blenis, 2004).

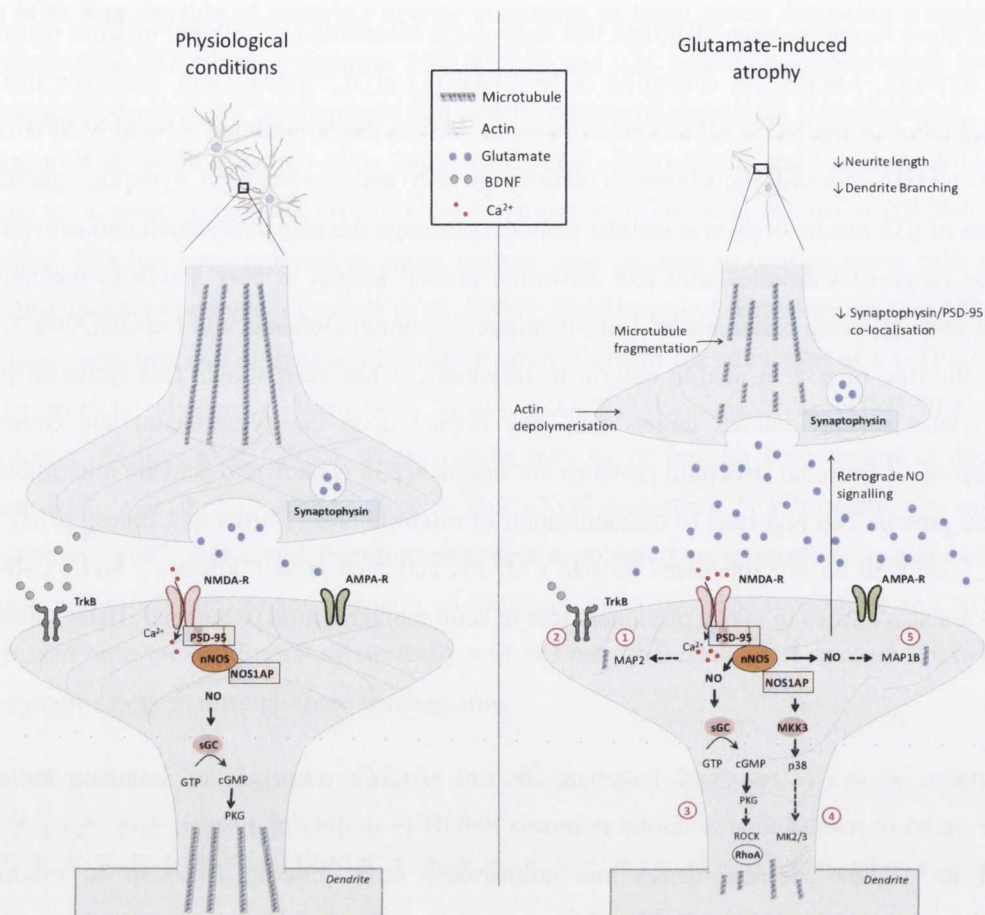


Figure 6.1: Potential mechanisms of glutamate- and NO- induced atrophy.

Left hand panel: In physiological conditions, basal activation of glutamate receptors at excitatory synapses is important for synaptic plasticity and the maintenance of normal neuronal function. A meshwork of actin filaments and microtubules form the cytoskeleton which acts to stabilise synapses and neurites and anchors proteins required for cell signalling at the synapse. Synapse number can be quantified by immunostaining for the co-localised expression of specific pre-synaptic and post-synaptic markers such as the pre-synaptic vesicle-associated protein, synaptophysin, and the post-synaptic protein, PSD-95. **Right hand panel:** Excessive glutamatergic stimulation results in decreased neurite outgrowth and a reduction in the co-localisation of synaptophysin and PSD-95. These effects can be linked to actin and microtubule destabilisation and fragmentation through several mechanisms acting both pre- and post-synaptically including: (1) NMDA-R-induced dephosphorylation of MAP2, (2) NO/cGMP/PKG-dependent downregulation of BDNF synthesis, (3) NO/cGMP/PKG-dependent activation of Rho/ROCK GTPase. Furthermore, NMDA-induced recruitment of NOS1AP links nNOS to the activation of the p38 MAPK pathway. (4) This pathway has been linked to cytoskeletal alterations via the p38 substrates MK2 and MK3 which are involved in actin reorganisation. (5) NO can also act independently of sGC to S-nitrosylate various proteins linked to the cytoskeleton such as MAP1B.

6.3 Neurotrophic and synaptogenic effects associated with NMDA-R/NO signalling inhibition

Increasing evidence suggests that NMDA-R antagonists and compounds which decrease NMDA-R/NO-related signalling possess novel neurotrophic properties through the activation of cellular signalling cascades associated with synaptogenesis and neurotrophic events (**Figure 6.2**). These effects have been particularly studied in relation to the antidepressant effects observed following ketamine administration, since compelling evidence from animal models suggests that the antidepressant-like properties of ketamine are associated with an increase in neurogenesis, dendritic length and spine density in the hippocampus and PFC, and a single dose of ketamine reverses the reduction in dendritic spine number which occurs following chronic stress paradigms (Duman and Li, 2012). The mechanism underlying these effects have been shown to include rapid activation of the mammalian target of rapamycin (mTOR) signalling pathway in the PFC of rats which was accompanied by increased levels and phosphorylation of key translation initiation factors including eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase (p70S6K), as well as increases in phosphorylated ERK and Akt. Importantly, the increases observed in these proteins and kinases occurs in line with increased translation of proteins associated with synapse formation including GluR1, synapsin and PSD-95, as well as an increased number and function of new spine synapses in the PFC (Li et al., 2010). Another study found the synaptogenic and antidepressant effects of ketamine involve inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) (which prevents its usual inhibitory influence on mTOR) and demonstrated that inhibition of GSK-3 potentiated the effects of a sub-effective dose of ketamine in these models (Liu et al., 2013). Ketamine's antidepressant-like effects are also reported to involve the synthesis of BDNF, with evidence showing that ketamine deactivates eukaryotic elongation factor 2 (eEF2) kinase which leads to dephosphorylation of eEF2 and augmentation of BDNF synthesis (Autry et al., 2011, Monteggia et al., 2013).

An important role for AMPA-Rs has been implicated in mediating the effects observed with ketamine administration as the AMPA-R antagonist, NBQX, attenuates ketamine's antidepressant-like effects in animal models of depression in rodents (Maeng et al., 2008, Koike et al., 2011). Interestingly, ketamine has been shown to facilitate an increase in pre-synaptic glutamate release and it has been suggested that ketamine may act to increase AMPA-R relative to NMDA-R throughput in critical neuronal circuits (Maeng and Zarate, 2007). Ketamine administration has also been shown to regulate hippocampal phosphorylation of the AMPA-R subunit GluR1 at the residue S845 (Maeng et al., 2008). This is of importance because NMDA-R-dependent regulation of this subunit is involved in the trafficking and membrane insertion properties of AMPA-R, and has been shown to occur via activation of cGMP-dependent kinase cGKII (Serulle et al., 2007) as well as through NO-mediated S-nitrosylation of GluR1

(Selvakumar et al., 2013). These results lend support to the suggestion that ketamine may alter AMPA-R trafficking properties, which may impact upon its effects on synaptogenesis.

These results are of interest for the current research since the neurotrophic effects observed in the present investigation with ketamine are similar to those observed through the use of downstream small molecule inhibition of the PSD-95/nNOS interaction using IC87201 and ZL006. These effects therefore may provide insights into the mechanism of action of IC87201 and ZL006 *in vivo*, which has not yet been established (Florio et al., 2009, Zhou et al., 2010, Bach et al., 2015). Prior research from our laboratory has shown that IC87201 and ZL006 display antidepressant-like effects in rodents (Doucet et al., 2013). The behavioural effects of IC87201 and ZL006 in rodent models are novel in that they are delayed and sustained following a single administration in the FST which is in contrast to the acute effects observed following conventional antidepressant administration. The delayed antidepressant-like effects may suggest that these compounds act by promoting adaptive neuroplastic changes in critical neuronal circuits, similar to those reported for ketamine. Recent evidence also demonstrates that the delayed efficacy of the NR2B/PSD-95 interacting peptide, Tat-HA-NR2B9c, in promoting functional recovery following stroke is associated with increased neurogenesis in the dentate gyrus and increased dendritic spine density of mature neurons in the motor cortex of rats in a rodent model of MCAO (Zhou et al., 2015). These authors report that ischaemia increases the levels and activity of S-nitrosylated cyclin dependent kinase 5 (Cdk5) in the hippocampus and found that these increases were reversed by TAT-HA-NR2B9c. S-nitrosylation of Cdk5 has previously been reported to contribute to NMDA-R-mediated synaptic spine loss caused by β -amyloid peptide (Qu et al., 2011), and the reduction in Cdk5 induced by TAT-HA-NR2B9c may therefore protect against these effects. Together, these findings suggest that the inhibition of elements downstream of the NMDA-R/NO signalling pathway may provide a means to enhance synaptogenic and/or neurotrophic effects which may contribute to their efficacy in neurological disorders associated with neuronal atrophy and synapse loss.

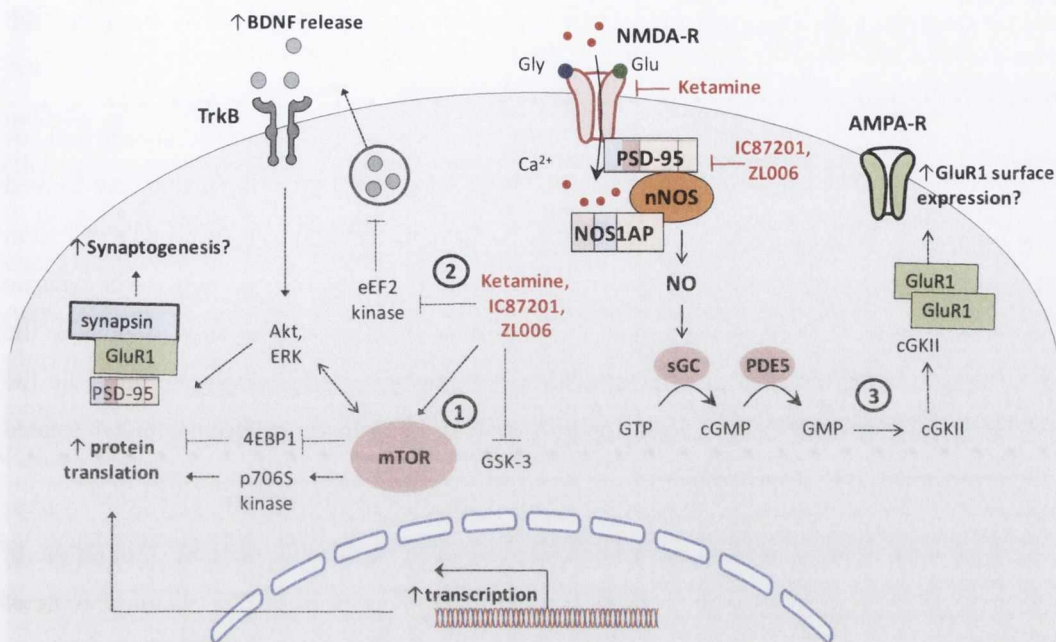


Figure 6.2: Mechanisms underlying the neurotrophic and synaptogenic effects associated with NMDA-R/NO signalling inhibition.

Ketamine is as a non-competitive NMDA-R antagonist that has been shown to increase dendritic spine formation and synaptogenesis in critical neuronal circuits concomitant to producing antidepressant behavioural effects in animal models. IC87201 and ZL006 are small molecule inhibitors of the PSD-95/nNOS interface which may also possess neurotrophic and synaptogenic properties. The potential mechanisms underlying these effects may include: ① Activation of the mTOR pathway, leading to phosphorylation and activation of 4EBP1, p70S6K and activated ERK and Akt. These effects occur in line with increased production of proteins associated with synapse formation such as PSD-95, GluR1 and synapsin. Ketamine also inhibits the phosphorylation of GSK-3 which prevents its usual inhibitory influence on mTOR. ② Ketamine has been shown to inhibit eEF2 kinase leading to dephosphorylation of eEF2 and augmentation of BDNF synthesis, a growth factor with well known roles in neuronal growth and synapse formation. ③ The antidepressant properties of ketamine are prevented by AMPA-R antagonism, indicating a role for AMPA-R in mediating ketamine's effects. NMDA-R-dependent regulation of AMPA-R trafficking has been shown to occur via NO-mediated S-nitrosylation of GluR1 and also by phosphorylation of GluR1 by downstream activation of cGMP-dependent kinase cGKII.

6.4 Potential mechanisms mediating the effects of L-AAA CM on neurite outgrowth and synaptic protein co-localisation

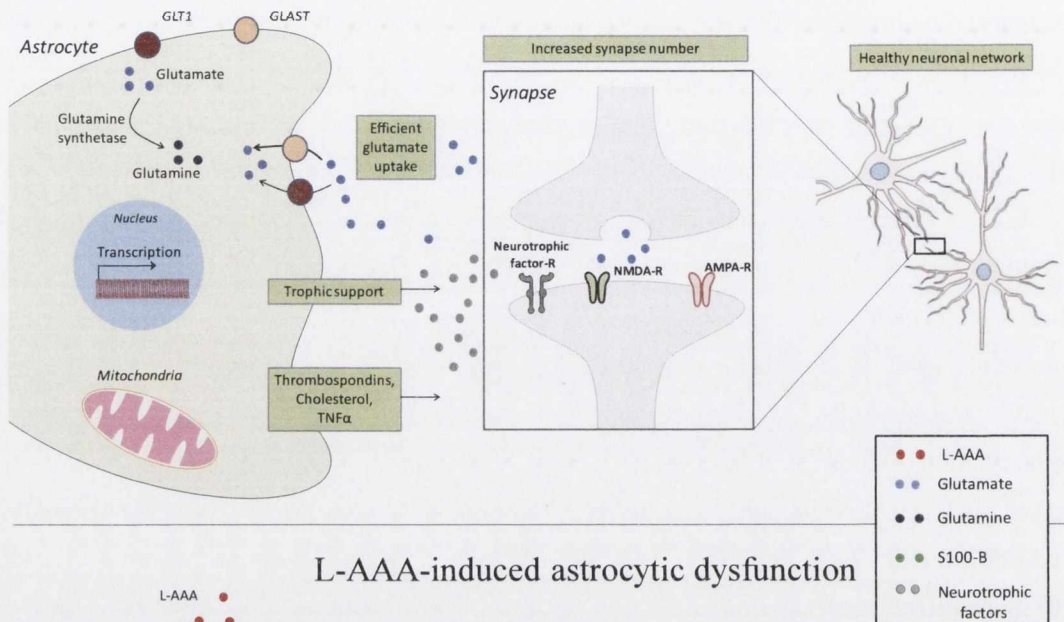
A further aim of this thesis was to investigate the effects of astrocytes on neuronal growth and co-localised expression of synaptic markers. The results of this research have shown that CM derived from astrocytes increases both neurite outgrowth as well as increasing the co-localised expression of synaptophysin and PSD-95 in primary cortical neurons, indicative of an increase in synapse number. Conversely, we demonstrate that L-AAA-induced astrocytic ablation abolishes the trophic effects of astrocyte CM on neurons. The potential mechanisms which may underlie the trophic effects of astrocyte CM, and the atrophic effects of CM derived from L-AAA treated astrocytes, are outlined in **Figure 6.3**.

The role that astrocytes play in the maintenance of neuronal health and the promotion of neurotrophic effects is firstly outlined in the top panel of Figure 6.3. In this regard, astrocytes express high affinity glutamate transporters that efficiently remove glutamate from the synapse as well as possessing the enzyme, glutamine synthetase, which is responsible for the conversion of glutamate to glutamine. Astrocytes therefore perform a crucial role in clearing glutamate from the synapse and prevent its potentially harmful accumulation in the extracellular space. The results presented in this thesis demonstrate that CM from astrocyte increases the co-localisation of synaptic protein markers, indicating that they increase synapse number, and this phenomenon is supported by prior research in the literature which has shown that astrocyte CM is sufficient to induce synaptogenesis in RGCs (Ullian et al., 2004). This synaptogenic effect has been attributed to astrocytic release of factors including the ECM molecules called thrombospondins (Christopherson et al., 2005), as well as cholesterol (Mauch et al., 2001, Pfrieger, 2003) and $\text{TNF}\alpha$ (Beattie et al., 2002). Previous research has also shown that CM derived from astrocytes is capable of providing neuronal protection against various detrimental stimuli including glutamate toxicity (Lu et al., 2015), corticosterone-induced damage (Zhu et al., 2006), methylmercury-induced neurotoxicity (Takemoto et al., 2015) and damage induced by reactive oxygen and nitrogen species (Tanaka et al., 1999), suggesting that astrocytes actively secrete soluble mediators which possess neuroprotective properties and promote healthy neuronal network maintenance.

In addition, the results presented in this thesis demonstrate that the treatment of astrocytes with the selective astrocyte toxin, L-AAA, abolished the trophic effects of astrocyte CM on synapse number and neurite outgrowth. Previous data in the literature has indicated that this toxin is uptaken via the glutamate transporters before accumulating intracellularly (Brown and Kretschmar, 1998). The mode of toxicity of L-AAA is believed to involve inhibition of glutamate transporters, and we provide evidence to support this by showing that L-AAA affects

the mRNA expression of these transporters. We also demonstrated that L-AAA increases the mRNA expression of S100B, an astrocyte-specific factor which is secreted in large amounts under pathological conditions such as Alzheimer's disease (Mrak and Griffin, 2001), depression (Schroeter et al., 2010) and stroke (Zhao and Rempe, 2010), and has been suggested to contribute to neuronal atrophy in these illnesses (Donato and Heizmann, 2010). L-AAA is also believed to exert toxicity in astrocytes through inhibition of glutamine synthetase - suggesting that L-AAA may lead to intracellular accumulation of glutamate, and inhibition of the enzyme γ -glutamylcysteine synthetase - indicating that L-AAA may contribute to a reduction in the synthesis of the important cellular antioxidant glutathione, thereby leading to oxidative stress (Olney et al., 1980, McBean, 1994). Further to this, we provide evidence indicating that L-AAA reduced mitochondrial oxygen consumption rates, indicative of reduced oxidative phosphorylation, reduced ATP and energy failure, which may impact upon essential astrocytic functions such as glutamate transporter activity for example. L-AAA has also been shown to lead to reduced protein synthesis in astrocytes after prolonged (>72h) exposure (Nishimura et al., 2000). Such effects may suggest that L-AAA leads to a reduction in the release of trophic factors and may contribute to neuronal atrophy through a lack of astrocytic trophic support.

Physiological conditions



L-AAA-induced astrocytic dysfunction

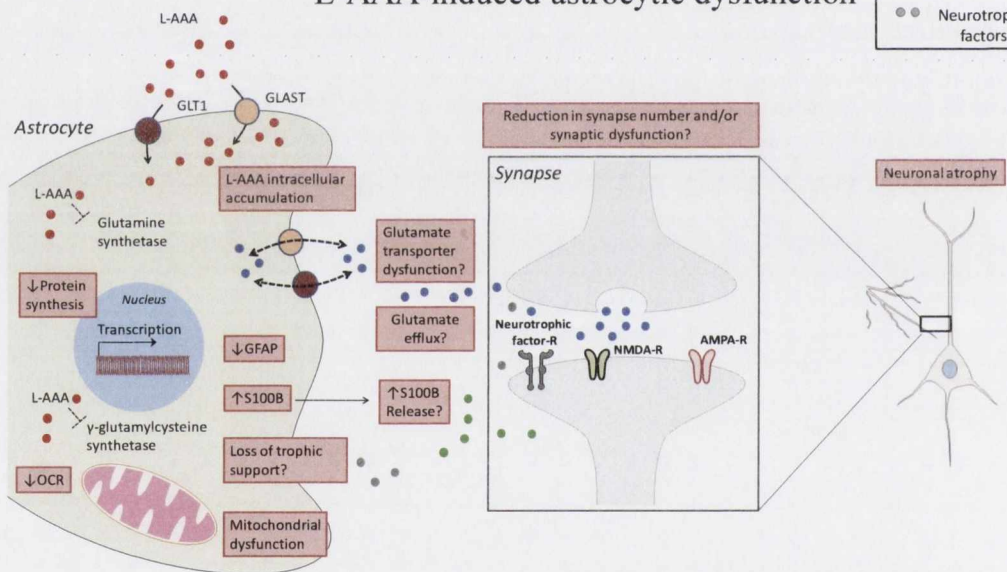


Figure 6.3: Potential mechanisms mediating the effects of healthy and L-AAA-induced dysfunctional astrocytes on neuronal atrophy. *Top panel:* In normal physiological conditions, astrocytes help to maintain healthy neuronal networks and regulate extracellular glutamate levels. They provide a CM which is trophic to neurons and leads to increases in synapse number, an effect attributed to the release of trophic and synaptogenic factors (positive effects of astrocytes on neurite growth are shown in green boxes). *Bottom panel:* In contrast, astrocytes treated with L-AAA produce a CM which abolishes the trophic effects observed with healthy astrocytes, leading to a reduction in synapse number and a decrease in neurite outgrowth. The mechanisms that may underlie L-AAA-induced astrocytic dysfunction and contribute to the production of atrophic CM are shown in red boxes and include: intracellular accumulation of L-AAA in astrocytes following uptake through the glutamate transporters GLT-1 and GLAST, inhibition of glutamine synthetase and γ-glutamylcysteine synthetase, reductions in GFAP and upregulation of S100B, inhibition of protein synthesis and a reduction in trophic support, and mitochondrial dysfunction and reduced oxygen consumption rates.

6.5 Future directions

6.5.1 Measurement of glutamate and NO concentrations and glutamate transporter activity in CM

Following on from the work presented in this thesis, it would be of interest to identify the biological factors contained in astrocyte CM which may account for the changes observed in neurite outgrowth and synaptic protein co-localisation in response to L-AAA. L-AAA may prevent or reduce the production of trophic factors produced basally by healthy astrocytes which promote neurite outgrowth, or alternatively, L-AAA may induce the production or release of certain factors that are detrimental for neurite outgrowth, such as glutamate or S100B for example. With regard to the first of these scenarios, previous research from our laboratory has shown that the treatment of astrocytes with noradrenaline resulted in increased expression of growth factors such as GDNF and FGF-2, and demonstrated that CM from these noradrenaline treated astrocytes lead to a concomitant increase in neurite outgrowth. We examined mRNA expression of growth factors in L-AAA treated astrocytes as we hypothesised that L-AAA may lead to reduced levels of such growth factors compared to control treated astrocytes, but no reductions in growth factor expression for GDNF or FGF-2 were demonstrated (data not shown). However, it may be of interest to examine the protein levels of growth factors in the CM as these may not necessarily correlate with mRNA expression profiles. This could be carried out using enzyme linked immunosorbent assays (ELISA) to quantify the concentration of growth factors in CM.

We have demonstrated that the treatment of astrocytes with L-AAA leads to alterations in the mRNA expression levels of glutamate transporters. Based on this evidence, it could be hypothesised that L-AAA impairs glutamate transporter function, and it may therefore be of interest to measure the activity of these transporters through functional assays. In addition, it would be of interest to analyse the levels of glutamate contained within L-AAA CM, since glutamate efflux is a well known feature of dysfunctional astrocytes (Anderson and Swanson, 2000). Moreover, we have shown that ketamine, TRIM and IC87201 were capable of attenuating L-AAA CM-induced reduction in neurite outgrowth. This may further allude to the potential novel neurotrophic properties of these compounds (see **Figure 6.2**) or it may suggest that L-AAA CM contributes to neurite atrophy through a mechanism of action involving NMDA-R/NO pathway activation (see **Figure 6.1**). It may be of interest therefore to determine glutamate and/or NO levels in the CM. The analysis of glutamate concentrations can be carried out through the use of colorimetric glutamate detection assays or through high-performance liquid chromatography (HPLC) coupled to fluorescence detection (Duan et al. 1999). Alternatively, glutamate uptake and release can be measured by liquid scintillation spectroscopy by assaying cells for their ability to

take up or release tritiated glutamate (Höltje et al. 2008). NO levels can be determined indirectly by the Griess assay which quantifies nitrite concentrations as a breakdown product of NO. Since our data help to confirm L-AAA as an astrocyte specific toxin, future experiments could further investigate the effects of L-AAA-induced astrocyte dysfunction on neuronal morphology by using an astrocyte-neuron co-culture model which would take into account the additional effects of astrocyte contact-mediated cues rather than observing the effects of astrocyte CM alone.

6.5.2 IC87201 and ZL006 in promoting neurotrophic and synaptogenic effects *in vivo*

The results of this thesis support the use of small molecular weight inhibitors at the PSD-95/nNOS interface and peptide inhibitors of the NOS1AP/nNOS interface for protection against glutamatergic based neuronal atrophy in cultured cortical neurons. Following on from the *in vitro* work presented here, it would be of interest to investigate whether IC87201 and ZL006 can enhance neurite growth and synapse formation in animal models of disease associated with neuronal atrophy and synapse loss. In this regard, it would be particularly relevant to examine whether the previously reported efficacy of IC87201 and ZL006 in animal models of depression (Doucet et al., 2013) involve increases in synaptogenesis or adaptive plastic changes in neuronal circuits. Investigating the potential of IC87201 and ZL006 in increasing dendritic arborisations and spine number *in vivo* could be determined using immunohistochemical approaches involving staining for a dendritic marker, such as MAP2, and analysing dendritic arborisations using Sholl analysis. In addition, the immunocytochemistry-based synapse assay used in the current research is also widely used for quantification of synapses *in vivo* (Ippolito and Eroglu, 2010) and could be adopted here for animal studies to directly investigate the translational potential for synaptogenic effects of IC87201 and ZL006 between *in vitro* and *in vivo* models. In addition to immunocytochemistry-based approaches, electrophysiological studies such as the patch-clamp technique would also be of interest to directly examine the activity and function of synapses *in vivo*.

Our work demonstrated that ketamine, TRIM and IC87201 were capable of attenuating the reduction in neurite outgrowth and reductions in co-localised synaptic proteins associated with CM-derived from L-AAA treated astrocytes. In animal models, L-AAA-induced astrocytic ablation in the PFC is sufficient to induce a depressive-like phenotype, and this L-AAA model has therefore been increasingly used as an animal model of depression (Banasr and Duman, 2008, Lima et al., 2014). Since ketamine, TRIM and IC87201 have all been shown to possess antidepressant-like effects; it may be of interest to investigate the potential for these compounds to attenuate L-AAA-induced depressive-like symptoms and/or to reverse the astroglial pathology and behavioural phenotype observed following chronic stress in animal models. In addition, an inbred genetic strain of rat, referred to as the Wistar-Kyoto (WKY) rat, represents an interesting model for investigating the effects of small molecule inhibitors at the PSD-95/nNOS interface.

The WKY rat is increasingly used as an animal model of depression since it displays spontaneous immobility in the FST, indicative of a state of behavioural despair, and has also been reported to exhibit reduced GFAP immunoreactive cells in several brain regions including the PFC (Gosselin et al., 2009). It may be of interest therefore, to assess the efficacy of ZL006 and IC87201 on behaviour in this rodent strain in order to investigate the potential for antidepressant-like efficacy in a rodent model with a genetically predisposed depressive-like state and astroglial pathology.

Finally, three dimensional magnetic resonance imaging (MRI) could also be employed to further characterise potential effects of ZL006 and IC87201 on structural alterations in animal models of depression. A large body of evidence from clinical MRI studies has indicated profound volume reductions in patients suffering from depression in several brain regions, most notably the PFC and hippocampus (Sheline, 2000, Hastings et al., 2004). MRI is a non-invasive imaging procedure which provides information on brain function and structure and can be applied in both pre-clinical and clinical environments. Since the ultimate goal in studying the effects of these compounds is to develop novel therapeutics for use in the clinic, information derived from pre-clinical MRI studies would be highly advantageous as it could provide new insights with powerful translational potential to a clinical setting.

6.5.3 NOS1AP interrupting peptides in promoting neurotrophic and synaptogenic effects *in vivo*

The potential of peptides interacting with the NOS1AP/nNOS interaction to increase neurite arborisation or synaptogenesis would also be of interest. For *in vivo* work using CPPs, the reverse, D form, of TAT-fused peptides are often used instead of the L form, since the D form is more resistant to proteases and possesses higher stability *in vivo* (Borsello et al., 2003). As such, D-TAT-GESV (100 ng) has been delivered intracerebroventricularly in rats, and has been shown to enter neurons in the brain after 6 h (Li et al., 2013). In this study, D-TAT-GESV was found to reduce p38 MAPK activation 30 min after hypoxia-ischaemia, and increase the amount of undamaged tissue compared to control treated (TAT-GASA) animals. The neuroprotective effects of this peptide were maintained up to one week following the ischaemic insult, similar to results observed using the p38 MAPK inhibitor, SB203580, indicating that NOS1AP/nNOS inhibition exerts sustained neuroprotective effects. Since IC87201 and ZL006 possess antidepressant-like effects in animal models, it would be interesting to determine whether TAT-GESV delivered into brain regions associated with mood disorders may also possess similar effects concomitant to potentially increasing neurite arborisation and synaptogenesis in critical neuronal circuits.

6.6 Concluding remarks

The findings of this thesis highlight the efficacy of selective inhibitors acting at the PSD-95/nNOS and NOS1AP/nNOS interface to protect against NMDA-induced atrophy in cultured

neurons. These results suggest that selective inhibition of protein interactions downstream of NMDA-R activation provides neuroprotection and represents an attractive drug target for the design of new therapeutics aimed at restoring synaptic connections and promoting neuronal remodelling in neurological illnesses associated with glutamate-induced neuronal atrophy. Future investigations in animal models are warranted in order to assess whether these inhibitors can promote neurotrophic effects *in vivo* and protect against neuronal atrophy in the absence of side effects commonly associated with direct NMDA-R antagonism or nNOS inhibition.

References

- Aarts M, Liu Y, Liu L, Besshoh S, Arundine M, Gurd JW, Wang Y-T, Salter MW, Tymianski M (2002) Treatment of ischemic brain damage by perturbing NMDA receptor- PSD-95 protein interactions. *Science* 298:846-850.
- Allen NJ (2013) Role of glia in developmental synapse formation. *Current opinion in neurobiology* 23:1027-1033.
- Allen NJ, Barres Ba (2005) Signaling between glia and neurons: focus on synaptic plasticity. *Current opinion in neurobiology* 15:542-548.
- Anderson CM, Swanson Ra (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32:1-14.
- Anderson MF, Blomstrand F, Blomstrand C, Eriksson PS, Nilsson M (2003) Astrocytes and stroke: networking for survival? *Neurochemical research* 28:293-305.
- Assouline JG, Bosch P, Lim R, Kim IS, Jensen R, Pantazis NJ (1987) Rat astrocytes and Schwann cells in culture synthesize nerve growth factor-like neurite-promoting factors. *Developmental Brain Research* 31:103-118.
- Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng P-f, Kavalali ET, Monteggia LM (2011) NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475:91-95.
- Bach A, Pedersen SW, Dorr LA, Vallon G, Ripoché I, Ducki S, Lian L-Y (2015) Biochemical investigations of the mechanism of action of small molecules ZL006 and IC87201 as potential inhibitors of the nNOS-PDZ/PSD-95-PDZ interactions. *Scientific reports* 5.
- Bakos J, Bacova Z, Grant SG, Castejon AM, Ostatnikova D (2015) Are Molecules Involved in Neuritogenesis and Axon Guidance Related to Autism Pathogenesis? *Neuromolecular medicine* 17:297-304.
- Bal-Price a, Brown GC (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21:6480-6491.
- Banasr M, Chowdhury GMI, Terwilliger R, Newton SS, Duman RS, Behar KL, Sanacora G (2010) Glial pathology in an animal model of depression: reversal of stress-induced cellular, metabolic and behavioral deficits by the glutamate-modulating drug riluzole. *Molecular psychiatry* 15:501-511.
- Banasr M, Duman RS (2008) Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. *Biological psychiatry* 64:863-870.
- Banasr M, Dwyer JM, Duman RS (2011) Cell atrophy and loss in depression: reversal by antidepressant treatment. *Current opinion in cell biology* 23:730-737.
- Banerjee A, Castro J, Sur M (2012) Rett syndrome: genes, synapses, circuits, and therapeutics. *Frontiers in psychiatry* 3:1-13.
- Barres BA (2008) The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron* 60:430-440.

- Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, Von Zastrow M, Beattie MS, Malenka RC (2002) Control of synaptic strength by glial TNF α . *Science* 295:2282-2285.
- Bechara C, Sagan S (2013) Cell-penetrating peptides: 20years later, where do we stand? *FEBS letters* 587:1693-1702.
- Bellamy TC, Garthwaite J (2002) The receptor-like properties of nitric oxide-activated soluble guanylyl cyclase in intact cells. *Molecular and cellular biochemistry* 230:165-176.
- Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH (2000) Antidepressant effects of ketamine in depressed patients. *Biological psychiatry* 47:351-354.
- Bicker G (2005) STOP and GO with NO: nitric oxide as a regulator of cell motility in simple brains. *Bioessays* 27:495-505.
- Bleakman D, Lodge D (1998) Neuropharmacology of AMPA and kainate receptors. *Neuropharmacology* 37:1187-1204.
- Blennow K, Hardy J, Zetterberg H (2012) The neuropathology and neurobiology of traumatic brain injury. *Neuron* 76:886-899.
- Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF (2001) Cell-permeable peptide inhibitors of JNK novel blockers of β -cell death. *Diabetes* 50:77-82.
- Borsello T, Bonny C (2004) Use of cell-permeable peptides to prevent neuronal degeneration. *Trends in molecular medicine* 10:239-244.
- Borsello T, Clarke PG, Hirt L, Vercelli A, Repici M, Schorderet DF, Bogousslavsky J, Bonny C (2003) A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nature medicine* 9:1180-1186.
- Bourgeron T (2009) A synaptic trek to autism. *Current opinion in neurobiology* 19:231-234.
- Boyle NT, Connor TJ (2007) MDMA ("Ecstasy") suppresses the innate IFN- γ response in vivo: a critical role for the anti-inflammatory cytokine IL-10. *European journal of pharmacology* 572:228-238.
- Brown B, Kang S, Gawelek K, Zacharias R, Anderson S, Turner C, Morris J (2015) In vivo and in vitro ketamine exposure exhibits a dose-dependent induction of activity-dependent neuroprotective protein in rat neurons. *Neuroscience* 290:31-40.
- Brown DR, Kretschmar Ha (1998) The gliotoxic mechanism of alpha-aminoadipic acid on cultured astrocytes. *Journal of neurocytology* 27:109-118.
- Brown GC, Bal-Price A (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Molecular neurobiology* 27:325-355.
- Brown GC, Borutaite V (2002) Nitric oxide inhibition of mitochondrial respiration and its role in cell death. *Free Radical Biology and Medicine* 33:1440-1450.
- Brzustowicz LM (2008) NOS1AP in schizophrenia. *Current psychiatry reports* 10:158-163.
- Buddle M, Eberhardt E, Ciminello LH, Levin T, Wing R, DiPasquale K, Raley-Susman KM (2003) Microtubule-associated protein 2 (MAP2) associates with the NMDA receptor and

is spatially redistributed within rat hippocampal neurons after oxygen-glucose deprivation. *Brain research* 978:38-50.

- Bustos FJ, Varela-Nallar L, Campos M, Henriquez B, Phillips M, Opazo C, Aguayo LG, Montecino M, Constantine-Paton M, Inestrosa NC, van Zundert B (2014) PSD95 Suppresses Dendritic Arbor Development in Mature Hippocampal Neurons by Occluding the Clustering of NR2B-NMDA Receptors. *PloS one* 9:e94037.
- Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AMG (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nature reviews Neuroscience* 8:766-775.
- Canossa M, Giordano E, Cappello S, Guarnieri C, Ferri S (2002) Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. *Proceedings of the National Academy of Sciences* 99:3282-3287.
- Cao J, Semenova MM, Solovyan VT, Han J, Coffey ET, Courtney MJ (2004) Distinct requirements for p38 α and c-Jun N-terminal kinase stress-activated protein kinases in different forms of apoptotic neuronal death. *Journal of Biological Chemistry* 279:35903-35913.
- Cao J, Viholainen JI, Dart C, Warwick HK, Leyland ML, Courtney MJ (2005) The PSD95-nNOS interface: a target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death. *The Journal of cell biology* 168:117-126.
- Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiology and Molecular Biology Reviews* 75:50-83.
- Carrel D, Du Y, Komlos D, Hadzimichalis NM, Kwon M, Wang B, Brzustowicz LM, Firestein BL (2009) NOS1AP regulates dendrite patterning of hippocampal neurons through a carboxypeptidase E-mediated pathway. *The Journal of Neuroscience* 29:8248-8258.
- Carrel D, Hernandez K, Kwon M, Mau C, Trivedi MP, Brzustowicz LM, Firestein BL (2015) Nitric Oxide Synthase 1 Adaptor Protein, a Protein Implicated in Schizophrenia, Controls Radial Migration of Cortical Neurons. *Biological psychiatry* 77:969-978.
- Casanova JR, Nishimura M, Owens JW, Swann JW (2012) Impact of seizures on developing dendrites: implications for intellectual developmental disabilities. *Epilepsia* 53:116-124.
- Centeno C, Repici M, Chatton J, Riederer B, Bonny C, Nicod P, Price M, Clarke P, Papa S, Franzoso G (2007) Role of the JNK pathway in NMDA-mediated excitotoxicity of cortical neurons. *Cell Death & Differentiation* 14:240-253.
- Chang IA, Kwon K-B, Park Y-C, Namgung U (2013) Permissive role of Cdc2 activity induced from astrocytes in neurite outgrowth. *Journal of neurochemistry* 1-11.
- Chang Y-F, Cauley RK, Chang J-D, Rao VV (1997) 1- α -Aminoadipate inhibits kynurenate synthesis in rat brain hippocampus and tissue culture. *Neurochemical research* 22:825-829.
- Charych EI, Akum BF, Goldberg JS, Jörnsten RJ, Rongo C, Zheng JQ, Firestein BL (2006) Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95. *The Journal of neuroscience* 26:10164-10176.

- Chen CJ, Liao SL, Kuo JS (2000) Gliotoxic action of glutamate on cultured astrocytes. *Journal of neurochemistry* 75:1557-1565.
- Christopherson KS, Hillier BJ, Lim WA, Brecht DS (1999) PSD-95 Assembles a Ternary Complex with the N-Methyl-D-aspartic Acid Receptor and a Bivalent Neuronal NO Synthase PDZ Domain. *Journal of Biological Chemistry* 274:27467-27473.
- Christopherson KS, Ullian EM, Stokes CCA, Mullen CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120:421-433.
- Chung IY, Benveniste EN (1990) Tumor necrosis factor- α production by astrocytes. Induction by lipopolysaccharide, IFN- γ , and IL-1 β . *The Journal of Immunology* 144:2999-3007.
- Chung W-S, Clarke LE, Wang GX, Stafford BK, Sher A, Chakraborty C, Joung J, Foo LC, Thompson A, Chen C (2013) Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* 504:394-400.
- Clarke LE, Barres BA (2013) Emerging roles of astrocytes in neural circuit development. *Nature reviews Neuroscience* 14:311-321.
- Cline HT (2001) Dendritic arbor development and synaptogenesis. *Current opinion in neurobiology* 11:118-126.
- Cohen-Cory S (2002) The developing synapse: construction and modulation of synaptic structures and circuits. *Science* 298:770-776.
- Contestabile A (2000) Roles of NMDA receptor activity and nitric oxide production in brain development. *Brain Research Reviews* 32:476-509.
- Cook DJ, Teves L, Tymianski M (2012) Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain. *Nature* 483:213-217.
- Corrêa SA, Eales KL (2012) The role of p38 MAPK and its substrates in neuronal plasticity and neurodegenerative disease. *Journal of signal transduction* 2012.
- Cotter DR, Pariante CM, Everall IP (2001) Glial cell abnormalities in major psychiatric disorders: the evidence and implications. *Brain research bulletin* 55:585-595.
- Courtney MJ, Li L-L, Lai YY (2014) Mechanisms of NOS1AP action on NMDA receptor-nNOS signaling. *Frontiers in cellular neuroscience* 8:252.
- Crotti L, Monti MC, Insolia R, Peljto A, Goosen A, Brink PA, Greenberg DA, Schwartz PJ, George AL (2009) NOS1AP is a genetic modifier of the long-QT syndrome. *Circulation* 120:1657-1663.
- Cuenda A, Rousseau S (2007) p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1773:1358-1375.
- da Silva JS, Dotti CG (2002) Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuriteogenesis. *Nature reviews Neuroscience* 3:694-704.

- Dawson VL, Dawson TM, London ED, Brecht DS, Snyder SH (1991) Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proceedings of the National Academy of Sciences* 88:6368-6371.
- Dawson VL, Dawson TM, Snyder H (1993) Mechanisms of Nitric Oxide-mediated Neurotoxicity in Primary Brain. *The Journal of neuroscience* 13:2651-2661.
- Day JS, O'Neill E, Cawley C, Aretz NK, Kilroy D, Gibney SM, Harkin A, Connor TJ (2014) Noradrenaline acting on astrocytic β 2-adrenoceptors induces neurite outgrowth in primary cortical neurons. *Neuropharmacology* 77:234-248.
- Day M, Wang Z, Ding J, An X, Ingham CA, Shering AF, Wokosin D, Ilijic E, Sun Z, Sampson AR (2006) Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nature neuroscience* 9:251-259.
- Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nature Reviews Neuroscience* 11:339-350.
- Dent EW, Gupton SL, Gertler FB (2011) The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harbor perspectives in biology* 3:a001800.
- Derkach Va, Oh MC, Guire ES, Soderling TR (2007) Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nature reviews Neuroscience* 8:101-113.
- Dhir A, Kulkarni SK (2011) Nitric oxide and major depression. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 24:125-131.
- DiazGranados N, Ibrahim L, Brutsche N, Ameli R, Henter ID, Luckenbaugh DA, Machado-Vieira R, Zarate Jr CA (2010) Rapid resolution of suicidal ideation after a single infusion of an NMDA antagonist in patients with treatment-resistant major depressive disorder. *The Journal of clinical psychiatry* 71:1605-1611.
- Dijkstra S, Bär P, Gispen W, Joosten E (1999) Selective stimulation of dendrite outgrowth from identified corticospinal neurons by homotopic astrocytes. *Neuroscience* 92:1331-1342.
- Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacological reviews* 51:7-61.
- Dlugos CA (2015) Ethanol-induced alterations in purkinje neuron dendrites in adult and aging rats: a review. *The Cerebellum* 14:466-473.
- Domin H, Szewczyk B, Woźniak M, Wawrzak-Wleciał A, Śmiałowska M (2014) Antidepressant-like effect of the mGluR5 antagonist MTEP in an astroglial degeneration model of depression. *Behavioural brain research* 273:23-33.
- Donato R, Heizmann CW (2010) S100B Protein in the Nervous System and Cardiovascular Apparatus in Normal and Pathological Conditions. *Cardiovascular psychiatry and neurology* 2010:929712.
- Doucet M, O'Toole E, Connor T, Harkin A (2015) Small-molecule inhibitors at the PSD-95/nNOS interface protect against glutamate-induced neuronal atrophy in primary cortical neurons. *Neuroscience* 301:421-438.

- Doucet MV, Harkin A, Dev KK (2012) The PSD-95/nNOS complex: new drugs for depression? *Pharmacology & therapeutics* 133:218-229.
- Doucet MV, Levine H, Dev KK, Harkin A (2013) Small molecule inhibitors at the PSD-95/nNOS interface have antidepressant-like properties in mice. *Neuropsychopharmacology* 8:1575-1584.
- Duman RS (2002) Pathophysiology of depression: the concept of synaptic plasticity. *European psychiatry : the journal of the Association of European Psychiatrists* 17 Suppl 3:306-310.
- Duman RS (2014) Pathophysiology of depression and innovative treatments: remodeling glutamatergic synaptic connections. *Dialogues in clinical neuroscience* 16:11.
- Duman RS, Aghajanian GK (2012) Synaptic dysfunction in depression: potential therapeutic targets. *Science* 338:68-72.
- Duman RS, Li N (2012) A neurotrophic hypothesis of depression: role of synaptogenesis in the actions of NMDA receptor antagonists. *Philosophical Transactions of the Royal Society B: Biological Sciences* 367:2475-2484.
- Duman RS, Li N, Liu R-J, Duric V, Aghajanian G (2012) Signaling pathways underlying the rapid antidepressant actions of ketamine. *Neuropharmacology* 62:35-41.
- Duman RS, Malberg J, Nakagawa S, D'Sa C (2000) Neuronal plasticity and survival in mood disorders. *Biological psychiatry* 48:732-739.
- Eastwood SL, Harrison PJ (2001) Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. *Brain research bulletin* 55:569-578.
- El-Husseini AE-D, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000) PSD-95 involvement in maturation of excitatory synapses. *Science* 290:1364-1368.
- Elfering SL, Sarkela TM, Giulivi C (2002) Biochemistry of mitochondrial nitric-oxide synthase. *Journal of Biological Chemistry* 277:38079-38086.
- Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicologic pathology* 35:495-516.
- Ernst AF, Gallo G, Letourneau PC, McLoon SC (2000) Stabilization of growing retinal axons by the combined signaling of nitric oxide and brain-derived neurotrophic factor. *The Journal of Neuroscience* 20:1458-1469.
- Eroglu C, Barres BA (2010) Regulation of synaptic connectivity by glia. *Nature* 468:223-231.
- Eshraghi AA, He J, Mou CH, Polak M, Zine A, Bonny C, Balkany TJ, Van De Water TR (2006) D-JNKI-1 treatment prevents the progression of hearing loss in a model of cochlear implantation trauma. *Otology & Neurotology* 27:504-511.
- Esquenazi S, Monnerie H, Kaplan P, Le Roux P (2002) BMP-7 and Excess Glutamate: Opposing Effects on Dendrite Growth from Cerebral Cortical Neurons in Vitro. *Experimental Neurology* 176:41-54.
- Ethell IM, Pasquale EB (2005) Molecular mechanisms of dendritic spine development and remodeling. *Progress in neurobiology* 75:161-205.

- Fang M, Jaffrey SR, Sawa a, Ye K, Luo X, Snyder SH (2000) Dexas1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. *Neuron* 28:183-193.
- Feng W, Zhang M (2009) Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. *Nature reviews Neuroscience* 10:87-99.
- Ferrick DA, Neilson A, Beeson C (2008) Advances in measuring cellular bioenergetics using extracellular flux. *Drug discovery today* 13:268-274.
- Fiala JC, Spacek J, Harris KM (2002) Dendritic spine pathology: cause or consequence of neurological disorders? *Brain research reviews* 39:29-54.
- Florio SK, Loh C, Huang SM, Iwamaye aE, Kitto KF, Fowler KW, Treiberg Ja, Hayflick JS, Walker JM, Fairbanks Ca, Lai Y (2009) Disruption of nNOS-PSD95 protein-protein interaction inhibits acute thermal hyperalgesia and chronic mechanical allodynia in rodents. *British journal of pharmacology* 158:494-506.
- Freudenberg F, Alttoa A, Reif A (2015) Neuronal nitric oxide synthase (NOS1) and its adaptor, NOS1AP, as a genetic risk factors for psychiatric disorders. *Genes, brain, and behavior* 14:46-63.
- Gallo G, Letourneau PC (2000) Neurotrophins and the Dynamic Regulation of the Neuronal Cytoskeleton. *Journal of neurobiology* 44:159-173.
- Gao Y-J, Ji R-R (2010) Targeting astrocyte signaling for chronic pain. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* 7:482-493.
- Gao Y-J, Zhang L, Samad OA, Suter MR, Yasuhiko K, Xu Z-Z, Park J-Y, Lind A-L, Ma Q, Ji R-R (2009) JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. *The Journal of Neuroscience* 29:4096-4108.
- Garthwaite J, Boulton C (1995) Nitric oxide signaling in the central nervous system. *Annual review of physiology* 57:683-706.
- Geschwind DH, Levitt P (2007) Autism spectrum disorders: developmental disconnection syndromes. *Current opinion in neurobiology* 17:103-111.
- Gigliucci V, Buckley KN, Nunan J, O'Shea K, Harkin A (2010) A role for serotonin in the antidepressant activity of NG-Nitro-L-arginine, in the rat forced swimming test. *Pharmacology Biochemistry and Behavior* 94:524-533.
- Glantz LA, Gilmore JH, Lieberman JA, Jarskog LF (2006) Apoptotic mechanisms and the synaptic pathology of schizophrenia. *Schizophrenia research* 81:47-63.
- Goldberg JL (2003) How does an axon grow? *Genes & development* 17:941-958.
- Gosselin R-D, Gibney S, O'Malley D, Dinan T, Cryan J (2009) Region specific decrease in glial fibrillary acidic protein immunoreactivity in the brain of a rat model of depression. *Neuroscience* 159:915-925.
- Gould E (2007) How widespread is adult neurogenesis in mammals? *Nature reviews Neuroscience* 8:481-488.
- Granato A, De Giorgio A (2014) Alterations of neocortical pyramidal neurons: turning points in the genesis of mental retardation. *Frontiers in pediatrics* 2:86.

- Greenwood SM, Connolly CN (2007) Dendritic and mitochondrial changes during glutamate excitotoxicity. *Neuropharmacology* 53:891-898.
- Guix F, Uribealago I, Coma M, Munoz F (2005) The physiology and pathophysiology of nitric oxide in the brain. *Progress in neurobiology* 76:126-152.
- Gutierrez H, Davies AM (2007) A fast and accurate procedure for deriving the Sholl profile in quantitative studies of neuronal morphology. *Journal of neuroscience methods* 163:24-30.
- Hadzimichalis NM, Previtera ML, Moreau MP, Li B, Lee GH, Dulencin AM, Matteson PG, Buyske S, Millonig JH, Brzustowicz LM (2010) NOS1AP protein levels are altered in BA46 and cerebellum of patients with schizophrenia. *Schizophrenia research* 124:248-250.
- Hall A, Lalli G (2010) Rho and Ras GTPases in axon growth, guidance, and branching. *Cold Spring Harbor perspectives in biology* 2:a001818.
- Halliday GM, Stevens CH, Hons B (2011) Glia : Initiators and Progressors of Pathology in Parkinson ' s Disease. 26:6-17.
- Halpain S, Greengard P (1990) Activation of NMDA receptors induces rapid dephosphorylation of the cytoskeletal protein MAP2. *Neuron* 5:237-246.
- Hamilton NB, Attwell D (2010) Do astrocytes really exocytose neurotransmitters? *Nature Reviews Neuroscience* 11:227-238.
- Han Q, Cai T, Tagle D, Robinson H, Li J (2009) Substrate specificity and structure of human aminoadipate aminotransferase/kynurenine aminotransferase II. *Bioscience Reports* 28:205-215.
- Hansen SM, Berezin V, Bock E (2008) Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin. *Cellular and molecular life sciences* 65:3809-3821.
- Hardingham N, Dachtler J, Fox K (2013) The role of nitric oxide in pre-synaptic plasticity and homeostasis. *Frontiers in cellular neuroscience* 7:00190.
- Harkin A, Connor TJ, Burns MP, Kelly JP (2004) Nitric oxide synthase inhibitors augment the effects of serotonin re-uptake inhibitors in the forced swimming test. *European Neuropsychopharmacology* 14:274-281.
- Harkin aJ, Bruce KH, Craft B, Paul Ia (1999) Nitric oxide synthase inhibitors have antidepressant-like properties in mice. 1. Acute treatments are active in the forced swim test. *European journal of pharmacology* 372:207-213.
- Harrison DC, Medhurst AD, Bond BC, Campbell CA, Davis RP, Philpott KL (2000) The use of quantitative RT-PCR to measure mRNA expression in a rat model of focal ischemia—caspase-3 as a case study. *Molecular brain research* 75:143-149.
- Harrison PJ (2004) The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. *Psychopharmacology* 174:151-162.
- Hashemian S, Marschinke F, af Bjerkén S, Strömberg I (2014) Degradation of proteoglycans affects astrocytes and neurite formation in organotypic tissue cultures. *Brain research* 1564:22-32.

- Hastings RS, Parsey RV, Oquendo MA, Arango V, Mann JJ (2004) Volumetric analysis of the prefrontal cortex, amygdala, and hippocampus in major depression. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology* 29:952-959.
- He CX, Portera-Cailliau C (2013) The trouble with spines in fragile X syndrome: density, maturity and plasticity. *Neuroscience* 251:120-128.
- He Y, Chen Z, Gong G, Evans A (2009) Neuronal networks in Alzheimer's disease. *The Neuroscientist* 15:333-350.
- He Y, Yu W, Baas PW (2002) Microtubule reconfiguration during axonal retraction induced by nitric oxide. *The Journal of neuroscience* 22:5982-5991.
- Hering H, Sheng M (2001) Dendritic spines: structure, dynamics and regulation. *Nature reviews Neuroscience* 2:880-888.
- Hess DT, Patterson SI, Smith DS, Skene JH (1993) Neuronal Growth cone collapse and inhibition of protein fatty acylation by nitric oxide. *Nature* 366:562-565.
- Hill MD, Martin RH, Mikulis D, Wong JH, Silver FL, Milot G, Clark WM, MacDonald RL, Kelly ME, Boulton M (2012) Safety and efficacy of NA-1 in patients with iatrogenic stroke after endovascular aneurysm repair (ENACT): a phase 2, randomised, double-blind, placebo-controlled trial. *The Lancet Neurology* 11:942-950.
- Hindley S, Juurlink BH, Gysbers JW, Middlemiss PJ, Herman Ma, Rathbone MP (1997) Nitric oxide donors enhance neurotrophin-induced neurite outgrowth through a cGMP-dependent mechanism. *Journal of neuroscience research* 47:427-439.
- Ho VM, Lee J-A, Martin KC (2011) The cell biology of synaptic plasticity. *Science* 334:623-628.
- Horn J, Limburg M (2001) Calcium Antagonists for Ischemic Stroke : A Systematic Review. *Stroke* 32:570-576.
- Hu J, Ferreira A, Eldik LV (1997) S 100B Induces Neuronal Cell Death Through Nitric Oxide Release from Astrocytes. *Journal of Neurochemistry* 69:2294-2301.
- Hu W, Guan L-S, Dang X-B, Ren P-Y, Zhang Y-L (2014) Small-molecule inhibitors at the PSD-95/nNOS interface attenuate MPP⁺-induced neuronal injury through Sirt3 mediated inhibition of mitochondrial dysfunction. *Neurochemistry international* 79:57-64.
- Huang C-C, Chan SH, Hsu K-S (2003) cGMP/protein kinase G-dependent potentiation of glutamatergic transmission induced by nitric oxide in immature rat rostral ventrolateral medulla neurons in vitro. *Molecular pharmacology* 64:521-532.
- Hurst R, Chowdhury R, Clark J (1996) Investigations into the mechanism of action of a novel nitric oxide generator on cellular respiration. *Journal of neurochemistry* 67:1200-1207.
- Iadecola C (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends in neurosciences* 20:132-139.
- Ikegaya Y, Kim J-A, Baba M, Iwatsubo T, Nishiyama N, Matsuki N (2001) Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA receptor activation: implication for a cellular defense against excitotoxicity. *Journal of cell science* 114:4083-4093.

- Ikonomidou C, Turski L (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury ? *Lancet Neurology* 1:383-386.
- Ippolito DM, Eroglu C (2010) Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. *Journal of visualized experiments* 2-9.
- Irwin SA, Galvez R, Greenough WT (2000) Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cerebral cortex* 10:1038-1044.
- Jaffrey SR, Snowman AM, Eliasson MJ, Cohen NA, Snyder SH (1998) CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. *Neuron* 20:115-124.
- Jane DE, Lodge D, Collingridge GL (2009) Neuropharmacology Kainate receptors : Pharmacology , function and therapeutic potential. *Neuropharmacology* 56:90-113.
- Jiang X, Georges PC, Li B, Du Y, Kutzing MK, Previtera ML, Langrana NA, Firestein BL (2007) Cell Growth in Response to Mechanical Stiffness is Affected by Neuron- Astroglia Interactions. *The Open Neuroscience Journal* 1:7-14.
- Johansson S, Strömberg I (2002) Guidance of dopaminergic neuritic growth by immature astrocytes in organotypic cultures of rat fetal ventral mesencephalon. *Journal of Comparative Neurology* 443:237-249.
- Johnson GL, Lapadat R (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298:1911-1912.
- Kamiguchi H, Lemmon V (2000) Recycling of the cell adhesion molecule L1 in axonal growth cones. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:3676-3686.
- Kao WL, Arking DE, Post W, Rea TD, Sotoodehnia N, Prineas RJ, Bishe B, Doan BQ, Boerwinkle E, Psaty BM (2009) Genetic variations in nitric oxide synthase 1 adaptor protein are associated with sudden cardiac death in US white community-based populations. *Circulation* 119:940-951.
- Kapoor A, Sekar RB, Hansen NF, Fox-Talbot K, Morley M, Pihur V, Chatterjee S, Brandimarto J, Moravec CS, Pulit SL (2014) An enhancer polymorphism at the cardiomyocyte intercalated disc protein NOS1AP locus is a major regulator of the QT interval. *The American Journal of Human Genetics* 94:854-869.
- Keshavan MS, Anderson S, Pettergrew JW (1994) Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? The Feinberg hypothesis revisited. *Journal of psychiatric research* 28:239-265.
- Kettenmann H, Kirchhoff F, Verkhratsky A (2013) Microglia: new roles for the synaptic stripper. *Neuron* 77:10-18.
- Kew JNC, Kemp JA (2005) Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology* 179:4-29.
- Khodosevich K, Monyer H (2010) Signaling involved in neurite outgrowth of postnatally born subventricular zone neurons in vitro. *BMC neuroscience* 1:18-29.

- Khurgel M, Koo aC, Ivy GO (1996) Selective ablation of astrocytes by intracerebral injections of alpha-aminoadipate. *Glia* 16:351-358.
- Kiryushko D, Berezin V, Bock E (2004) Regulators of Neurite Outgrowth: Role of Cell Adhesion Molecules. *Annals of the New York Academy of Sciences* 1014:140-154.
- Koike H, Iijima M, Chaki S (2011) Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behavioural brain research* 224:107-111.
- Koleske AJ (2013) Molecular mechanisms of dendrite stability. *Nature Reviews Neuroscience* 14:536-550.
- Konradi C, Heckers S (2003) Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. *Pharmacology & therapeutics* 97:153-179.
- Kulkarni VA, Firestein BL (2012) The dendritic tree and brain disorders. *Molecular and Cellular Neuroscience* 50:10-20.
- Kuma Y, Sabio G, Bain J, Shpiro N, Márquez R, Cuenda A (2005) BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *Journal of Biological Chemistry* 280:19472-19479.
- Kuo TY, Chen CY, Hsueh YP (2010) Bcl11A/CTIP1 mediates the effect of the glutamate receptor on axon branching and dendrite outgrowth. *Journal of neurochemistry* 114:1381-1392.
- Kurt MA, Kafa MI, Dierssen M, Davies DC (2004) Deficits of neuronal density in CA1 and synaptic density in the dentate gyrus, CA3 and CA1, in a mouse model of Down syndrome. *Brain research* 1022:101-109.
- Labby KJ, Xue F, Kraus JM, Ji H, Mataka J, Li H, Martásek P, Roman LJ, Poulos TL, Silverman RB (2012) Intramolecular hydrogen bonding: a potential strategy for more bioavailable inhibitors of neuronal nitric oxide synthase. *Bioorganic & medicinal chemistry* 20:2435-2443.
- Lee W-H, Xu Z, Ashpole NM, Hudmon A, Kulkarni PM, Thakur GA, Lai YY, Hohmann AG (2015) Small molecule inhibitors of PSD95-nNOS protein-protein interactions as novel analgesics. *Neuropharmacology*.
- Lefrançois T, Fages C, Peschanski M, Tardy M (1997) Neuritic outgrowth associated with astroglial phenotypic changes induced by antisense glial fibrillary acidic protein (GFAP) mRNA in injured neuron-astrocyte cocultures. *The Journal of neuroscience* 17:4121-4128.
- Li GN, Liu J, Hoffman-Kim D (2008) Multi-molecular gradients of permissive and inhibitory cues direct neurite outgrowth. *Annals of biomedical engineering* 36:889-904.
- Li L-L, de Mera RMM-F, Chen J, Ba W, Kasri NN, Zhang M, Courtney MJ (2015) Unexpected Heterodivalent Recruitment of NOS1AP to nNOS Reveals Multiple Sites for Pharmacological Intervention in Neuronal Disease Models. *The Journal of Neuroscience* 35:7349-7364.
- Li L-L, Ginet V, Liu X, Vergun O, Tuittila M, Mathieu M, Bonny C, Puyal J, Truttmann AC, Courtney MJ (2013) The nNOS-p38MAPK pathway is mediated by NOS1AP during neuronal death. *The Journal of Neuroscience* 33:8185-8201.

- Li N, Lee B, Liu R-J, Banasr M, Dwyer JM, Iwata M, Li X-Y, Aghajanian G, Duman RS (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329:959-964.
- Li N, Liu R-J, Dwyer JM, Banasr M, Lee B, Son H, Li X-Y, Aghajanian G, Duman RS (2011) Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biological psychiatry* 69:754-761.
- Lima A, Sardinha VM, Oliveira aF, Reis M, Mota C, Silva Ma, Marques F, Cerqueira JJ, Pinto L, Sousa N, Oliveira JF (2014) Astrocyte pathology in the prefrontal cortex impairs the cognitive function of rats. *Molecular psychiatry* 1-8.
- Liu D, Smith CL, Barone FC, Ellison JA, Lysko PG, Li K, Simpson IA (1999) Astrocytic demise precedes delayed neuronal death in focal ischemic rat brain. *Brain research Molecular brain research* 68:29-41.
- Liu R-J, Fuchikami M, Dwyer JM, Lepack AE, Duman RS, Aghajanian GK (2013) GSK-3 inhibition potentiates the synaptogenic and antidepressant-like effects of subthreshold doses of ketamine. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 38:2268-2277.
- Liu R, Wang Z, Gou L, Xu H (2015) A cortical astrocyte subpopulation inhibits axon growth in vitro and in vivo. *Molecular medicine reports* 12:2598-2606.
- Lowery LA, Van Vactor D (2009) The trip of the tip: understanding the growth cone machinery. *Nature reviews Molecular cell biology* 10:332-343.
- Lu X, Al-Aref R, Zhao D, Shen J, Yan Y, Gao Y (2015) Astrocyte-conditioned medium attenuates glutamate-induced apoptotic cell death in primary cultured spinal cord neurons of rats. *Neurological research* 37:803-808.
- Luo C-X, Lin Y-H, Qian X-D, Tang Y, Zhou H-H, Jin X, Ni H-Y, Zhang F-Y, Qin C, Li F (2014) Interaction of nNOS with PSD-95 Negatively Controls Regenerative Repair after Stroke. *The Journal of Neuroscience* 34:13535-13548.
- Luo L (2002) Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annual review of cell and developmental biology* 18:601-635.
- Lynch ED, Kil J (2005) Compounds for the prevention and treatment of noise-induced hearing loss. *Drug discovery today* 10:1291-1298.
- MacDonald J, Wojtowicz J (1982) The effects of L-glutamate and its analogues upon the membrane conductance of central murine neurones in culture. *Canadian journal of physiology and pharmacology* 60:282-296.
- Maeng S, Zarate Ca (2007) The role of glutamate in mood disorders: results from the ketamine in major depression study and the presumed cellular mechanism underlying its antidepressant effects. *Current psychiatry reports* 9:467-474.
- Maeng S, Zarate Ca, Du J, Schloesser RJ, McCammon J, Chen G, Manji HK (2008) Cellular mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biological psychiatry* 63:349-352.
- Malarkey EB, Parpura V (2009) Mechanisms of glutamate release from astrocytes. *Neurochemistry international* 52:142-154.

- Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annual review of neuroscience* 25:103-126.
- Maragakis NJ, Rothstein JD (2006) Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nature clinical practice Neurology* 2:679-689.
- Martin S, Grimwood P, Morris R (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. *Annual review of neuroscience* 23:649-711.
- Matsutani S, Yamamoto N (1998) GABAergic neuron-to-astrocyte signaling regulates dendritic branching in coculture. *Journal of neurobiology* 37:251-264.
- Mattson M, Dou P, Kater B (1988) Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *The Journal of neuroscience* 8:2087-2100.
- Mattson MP (2008) Glutamate and neurotrophic factors in neuronal plasticity and disease. *Annals of the New York Academy of Sciences* 1144:97-112.
- Mauch DH, Nägler K, Schumacher S, Göritz C, Müller EC, Otto a, Pfrieder FW (2001) CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294:1354-1357.
- McAllister AK (2007) Dynamic aspects of synapse formation. *Annual review of neuroscience* 30:425.
- McBean GJ (1994) Inhibition of the glutamate transporter and glial enzymes in rat striatum by the gliotoxin, alpha aminoadipate. *British journal of pharmacology* 113:536-540.
- McEwen BS, Morrison JH (2013) The brain on stress: vulnerability and plasticity of the prefrontal cortex over the life course. *Neuron* 79:16-29.
- McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *The Journal of neuroscience* 11:3398-3411.
- McKinney RA, Capogna M, Dürer R, Gähwiler BH, Thompson SM (1999) Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nature neuroscience* 2:44-49.
- Mehta A, Prabhakar M, Kumar P, Deshmukh R, Sharma P (2013) Excitotoxicity: bridge to various triggers in neurodegenerative disorders. *European journal of pharmacology* 698:6-18.
- Mei L, Xiong W-C (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nature reviews Neuroscience* 9:437-452.
- Metzger F, Wiese S, Sendtner M (1998) Effect of glutamate on dendritic growth in embryonic rat motoneurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18:1735-1742.
- Meyer MP, Smith SJ (2006) Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *The Journal of neuroscience* 26:3604-3614.
- Minichiello L (2009) TrkB signalling pathways in LTP and learning. *Nature Reviews Neuroscience* 10:850-860.

- Molofsky AV, Krencik R, Krenick R, Ullian EM, Tsai H-h, Deneen B, Richardson WD, Barres Ba, Rowitch DH (2012) Astrocytes and disease: a neurodevelopmental perspective. *Genes & development* 26:891-907.
- Molz S, Decker H, Dal-Cim T, Cremonez C, Cordova FM, Leal RB, Tasca CI (2008) Glutamate-induced toxicity in hippocampal slices involves apoptotic features and p38MAPK signaling. *Neurochemical research* 33:27-36.
- Monnerie H, Shashidhara S, Le Roux PD (2003) Effect of excess extracellular glutamate on dendrite growth from cerebral cortical neurons at 3 days in vitro: Involvement of NMDA receptors. *Journal of neuroscience research* 74:688-700.
- Monteggia LM, Gideons E, Kavalali ET (2013) The role of eukaryotic elongation factor 2 kinase in rapid antidepressant action of ketamine. *Biological psychiatry* 73:1199-1203.
- Montoliu C, Llansola M, Kosenko E, Corbalán R, Felipo V (1999) Role of cyclic GMP in glutamate neurotoxicity in primary cultures of cerebellar neurons. *Neuropharmacology* 38:1883-1891.
- Moreno-López B, Sunico CR, González-Forero D (2011) NO orchestrates the loss of synaptic boutons from adult "sick" motoneurons: modeling a molecular mechanism. *Molecular neurobiology* 43:41-66.
- Moskowitz MA, Lo EH, Iadecola C (2010) The science of stroke: mechanisms in search of treatments. *Neuron* 67:181-198.
- Mrak RE, Griffin WST (2001) The role of activated astrocytes and of the neurotrophic cytokine S100B in the pathogenesis of Alzheimer 's disease. 22:915-922.
- Muir KW (2006) Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. *Current opinion in pharmacology* 6:53-60.
- Nägler K, Mauch DH, Pfrieger FW (2001) Glia-derived signals induce synapse formation in neurones of the rat central nervous system. *The Journal of physiology* 533:665-679.
- Niell CM, Meyer MP, Smith SJ (2004) In vivo imaging of synapse formation on a growing dendritic arbor. *Nature neuroscience* 7:254-260.
- Nimchinsky Ea, Sabatini BL, Svoboda K (2002) Structure and function of dendritic spines. *Annual review of physiology* 64:313-353.
- Nishimoto S, Nishida E (2006) MAPK signalling: ERK5 versus ERK1/2. *EMBO reports* 7:782-786.
- Nishimura R, Santos D, Fu S-T, Dwyer B (2000) Induction of cell death by L-alpha-aminoadipic acid exposure in cultured rat astrocytes: relationship to protein synthesis. *Neurotoxicology* 21:313-320.
- O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *European Journal of Biochemistry* 267:5421-5426.
- Olney JW, de Gubareff T, Collins JF (1980) Stereospecificity of the gliotoxic and anti-neurotoxic actions of alpha-aminoadipate. *Neuroscience Letters* 19:277-282.

- Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiological reviews* 87:315-424.
- Palop JJ, Mucke L (2010) Amyloid- β -induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nature neuroscience* 13:812-818.
- Panickar KS, Norenberg MD (2005) Astrocytes in cerebral ischemic injury: morphological and general considerations. *Glia* 50:287-298.
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine reviews* 22:153-183.
- Perea G, Navarrete M, Araque A (2009) Tripartite synapses: astrocytes process and control synaptic information. *Trends in neurosciences* 32:421-431.
- Pfriege FW (2003) Role of cholesterol in synapse formation and function. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1610:271-280.
- Pfriege FW, Barres BA (1997) Synaptic Efficacy Enhanced by Glial Cells in Vitro. *Science* 277:1684-1687.
- Piao CS, Kim JB, Han PL, Lee JK (2003) Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. *Journal of neuroscience research* 73:537-544.
- Podestá MF, Yam P, Codagnone MG, Uccelli NA, Colman D, Reinés A (2014) Distinctive PSA-NCAM and NCAM Hallmarks in Glutamate-Induced Dendritic Atrophy and Synaptic Disassembly. *PloS one* 9:e108921.
- Polleux F, Morrow T, Ghosh a (2000) Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* 404:567-573.
- Polleux F, Snider W (2010) Initiating and growing an axon. *Cold Spring Harbor perspectives in biology* 2:a001925.
- Popoli M, Yan Z, McEwen BS, Sanacora G (2012) The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nature reviews Neuroscience* 13:22-37.
- Previtera ML, Langhammer CG, Langrana Na, Firestein BL (2010) Regulation of dendrite arborization by substrate stiffness is mediated by glutamate receptors. *Annals of biomedical engineering* 38:3733-3743.
- Qu J, Nakamura T, Cao G, Holland EA, McKercher SR, Lipton SA (2011) S-Nitrosylation activates Cdk5 and contributes to synaptic spine loss induced by β -amyloid peptide. *Proceedings of the National Academy of Sciences* 108:14330-14335.
- Rajkowska G, Miguel-Hidalgo JJ (2007) Gliogenesis and glial pathology in depression. *CNS & neurological disorders drug targets* 6:219-233.
- Rampersad SN (2012) Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors* 12:12347-12360.
- Ranscht B (2000) Cadherins: molecular codes for axon guidance and synapse formation. *International Journal of Developmental Neuroscience* 18:643-651.

- Richier L, Williton K, Clattenburg L, Colwill K, O'Brien M, Tsang C, Kolar A, Zinck N, Metalnikov P, Trimble WS, Krueger SR, Pawson T, Fawcett JP (2010) The Nitric Oxide Synthase Adaptor Protein (NOS1AP) Associates with Scribble and Regulates Dendritic Spine Development. *The Journal of neuroscience* 30:4796-4805.
- Rigato F, Garwood J, Heck N, Faivre-sarrailh C (2002) Tenascin-C Promotes Neurite Outgrowth of Embryonic Hippocampal Neurons through the Alternatively Spliced Fibronectin Type III BD Domains via Activation of the Cell Adhesion Molecule F3 / Contactin. *The Journal of neuroscience* 22:6596-6609.
- Robson MJ, Elliott M, Seminerio MJ, Matsumoto RR (2012) Evaluation of sigma (σ) receptors in the antidepressant-like effects of ketamine in vitro and in vivo. *European neuropsychopharmacology* 22:308-317.
- Rönicke R, Mikhaylova M, Rönicke S, Meinhardt J, Schröder UH, Fändrich M, Reiser G, Kreutz MR, Reymann KG (2011) Early neuronal dysfunction by amyloid β oligomers depends on activation of NR2B-containing NMDA receptors. *Neurobiology of aging* 32:2219-2228.
- Roqué PJ, Guizzetti M, Costa LG (2014) Synaptic structure quantification in cultured neurons. *Current protocols in toxicology* 60:DOI:10.1002/0471140856.tx0471141222s0471140860.
- Roux PP, Blenis J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and molecular biology reviews* 68:320-344.
- Sanacora G, Treccani G, Popoli M (2012) Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* 62:63-77.
- Sanchez C, Diaz-Nido J, Avila J (2000) Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. *Progress in neurobiology* 61:133-168.
- Santos A, Martínez-Ruiz A, Araújo I (2014) S-nitrosation and neuronal plasticity. *British journal of pharmacology*.
- Scannevin RH, Huganir RL (2000) Postsynaptic organization and regulation of excitatory synapses. *Nature reviews Neuroscience* 1:133-141.
- Scheiffele P (2003) Cell-cell signaling during synapse formation in the CNS. *Annual review of neuroscience* 26:485-508.
- Scholz J, Woolf CJ (2007) The neuropathic pain triad: neurons, immune cells and glia. *Nature neuroscience* 10:1361-1368.
- Schroeter ML, Abdul-Khaliq H, Diefenbacher A, Blasig IE (2002) S100B is increased in mood disorders and may be reduced by antidepressive treatment. *Neuroreport* 13:1675-1678.
- Schroeter ML, Abdul-Khaliq H, Sacher J, Steiner J, Blasig IE, Mueller K (2010) Mood disorders are glial disorders: evidence from in vivo studies. *Cardiovascular psychiatry and neurology* 2010:780645.
- Scott EK, Luo L (2001) How do dendrites take their shape? *Nature neuroscience* 4:359-365.

- Selvakumar B, Jenkins Ma, Hussain NK, Hugarir RL, Traynelis SF, Snyder SH (2013) S-nitrosylation of AMPA receptor GluA1 regulates phosphorylation, single-channel conductance, and endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* 110:1077-1082.
- Serulle Y, Zhang S, Ninan I, Puzzo D, McCarthy M, Khatri L, Arancio O, Ziff EB (2007) A GluR1-cGKII interaction regulates AMPA receptor trafficking. *Neuron* 56:670-688.
- Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL (2007) Natural oligomers of the Alzheimer amyloid- β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *The Journal of neuroscience* 27:2866-2875.
- Sheline YI (2000) 3D MRI studies of neuroanatomic changes in unipolar major depression: the role of stress and medical comorbidity. *Biological psychiatry* 48:791-800.
- Shelly M, Lim BK, Cancedda L, Heilshorn SC, Gao H, Poo M-m (2010) Local and long-range reciprocal regulation of cAMP and cGMP in axon/dendrite formation. *Science* 327:547-552.
- Sholl DA (1953) Dendritic organisation in the neurons of the visual and motor cortices of the cat. *Journal of Anatomy* 87:387-406.
- Shortland PJ, Leinster VH, White W, Robson LG (2006) Riluzole promotes cell survival and neurite outgrowth in rat sensory neurones in vitro. *European Journal of Neuroscience* 24:3343-3353.
- Silva SL, Vaz AR, Diógenes MJ, van Rooijen N, Sebastião AM, Fernandes A, Silva RFM, Brites D (2012) Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycochenodeoxycholic acid and interleukin-10. *Neuropharmacology* 62:2398-2408.
- Smith-Thomas LC, Fok-Seang J, Stevens J, Du JS, Muir E, Faissner a, Geller HM, Rogers JH, Fawcett JW (1994) An inhibitor of neurite outgrowth produced by astrocytes. *Journal of cell science* 107 (Pt 6:1687-1695.
- Snow D, Beller J (2014) Proteoglycans: Road Signs for Neurite Outgrowth. *Neural Regeneration Research* 9:343-355.
- Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J (1990) Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Experimental neurology* 109:111-130.
- Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 11:400-407.
- Sofroniew MV (2009) Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in neurosciences* 32:638-647.
- Sofroniew MV, Vinters HV (2010) Astrocytes: biology and pathology. *Acta neuropathologica* 119:7-35.
- Soriano FX, Martel M-A, Papadia S, Vaslin A, Baxter P, Rickman C, Forder J, Tymianski M, Duncan R, Aarts M (2008) Specific targeting of pro-death NMDA receptor signals with

- differing reliance on the NR2B PDZ ligand. *The Journal of Neuroscience* 28:10696-10710.
- Southam E, Charles SL, Garthwaite J (1996) The nitric oxide-cyclic GMP pathway and synaptic plasticity in the rat superior cervical ganglion. *British journal of pharmacology* 119:527-532.
- Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF- α . *Nature* 440:1054-1059.
- Stroissnigg H, Trančíková A, Descovich L, Fuhrmann J, Kutschera W, Kostan J, Meixner A, Nothias F, Propst F (2007) S-nitrosylation of microtubule-associated protein 1B mediates nitric-oxide-induced axon retraction. *Nature Cell Biology* 9:1035-1045.
- Sun H-S, Doucette TA, Liu Y, Fang Y, Teves L, Aarts M, Ryan CL, Bernard PB, Lau A, Forder JP (2008) Effectiveness of PSD95 inhibitors in permanent and transient focal ischemia in the rat. *Stroke* 39:2544-2553.
- Sunico CR, González-Forero D, Domínguez G, García-Verdugo JM, Moreno-López B (2010) Nitric oxide induces pathological synapse loss by a protein kinase G-, Rho kinase-dependent mechanism preceded by myosin light chain phosphorylation. *The Journal of Neuroscience* 30:973-984.
- Swaab D, Hofman M, Lucassen P, Salehi A, Uylings H (1994) Neuronal atrophy, not cell death, is the main hallmark of Alzheimer's disease. *Neurobiology of aging* 15:369-371.
- Takano T, Oberheim N, Cotrina ML, Nedergaard M (2009) Astrocytes and ischemic injury. *Stroke* 40:S8-12.
- Takemoto T, Ishihara Y, Ishida A, Yamazaki T (2015) Neuroprotection elicited by nerve growth factor and brain-derived neurotrophic factor released from astrocytes in response to methylmercury. *Environmental toxicology and pharmacology* 40:199-205.
- Tanaka J, Toku K, Zhang BO, Ishihara KEN (1999) Astrocytes Prevent Neuronal Death Induced by Reactive Oxygen and Nitrogen Species. *Glia* 96:85-96.
- Tata DA, Anderson BJ (2010) The effects of chronic glucocorticoid exposure on dendritic length, synapse numbers and glial volume in animal models: Implications for hippocampal volume reductions in depression. *Physiology & Behavior* 186-193.
- Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. *Nature reviews Neuroscience* 5:173-183.
- Tsai MJ, Chang YF, Schwarcz R, Brookes N (1996) Characterization of L-alpha-aminoadipic acid transport in cultured rat astrocytes. *Brain research* 741:166-173.
- Tseng C-Y, Firestein BL (2011) The role of PSD-95 and cypin in morphological changes in dendrites following sublethal NMDA exposure. *The Journal of Neuroscience* 31:15468-15480.
- Tuinstra HM, Ducommun MM, Briley WE, Shea LD (2013) Gene delivery to overcome astrocyte inhibition of axonal growth: an in vitro model of the glial scar. *Biotechnology and bioengineering* 110:947-957.

- Ullian EM, Christopherson KS, Barres Ba (2004) Role for glia in synaptogenesis. *Glia* 47:209-216.
- Ullian EM, Sapperstein SK, Christopherson KS, Barres Ba (2001) Control of synapse number by glia. *Science* 291:657-661.
- Vallance P, Leiper J (2002) Blocking NO synthesis: how, where and why? *Nature Reviews Drug Discovery* 1:939-950.
- Vaslin A, Naegele-Tollardo S, Puyal J, Clarke PG (2011) Excitotoxicity-induced endocytosis mediates neuroprotection by TAT-peptide-linked JNK inhibitor. *Journal of neurochemistry* 119:1243-1252.
- Vécsei L, Szalárdy L, Fülöp F, Toldi J (2013) Kynurenines in the CNS: recent advances and new questions. *Nature reviews Drug discovery* 12:64-82.
- Verkhatsky A, Olabarria M, Noristani HN, Yeh C-Y, Rodriguez JJ (2010) Astrocytes in Alzheimer's disease. *Neurotherapeutics* 7:399-412.
- Wang Y-F, Hatton GI (2009) Astrocytic plasticity and patterned oxytocin neuronal activity: dynamic interactions. *The Journal of Neuroscience* 29:1743-1754.
- Watkins LR, Maier SF (2003) Glia: a novel drug discovery target for clinical pain. *Nature reviews Drug discovery* 2:973-985.
- Wegener G, Volke V (2010) Nitric Oxide Synthase Inhibitors as Antidepressants. *Pharmaceuticals* 3:273-299.
- Willard SS, Koochekpour S (2013) Glutamate, Glutamate Receptors, and Downstream Signaling Pathways. *International journal of biological sciences* 9:948-959.
- Williams EJ, Walsh FS, Doherty P (1994) Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. *The Journal of cell biology* 124:1029-1037.
- Wilson MT, Keith CH (1998) Glutamate modulation of dendrite outgrowth: alterations in the distribution of dendritic microtubules. *Journal of neuroscience research* 52:599-611.
- Wu H-Q, Ungerstedt U, Schwarcz R (1995) l- α -Aminoadipic acid as a regulator of kynurenic acid production in the hippocampus: a microdialysis study in freely moving rats. *European Journal of Pharmacology* 281:55-61.
- Xu B, Wratten N, Charych EI, Buyske S, Firestein BL, Brzustowicz LM (2005) Increased expression in dorsolateral prefrontal cortex of CAPON in schizophrenia and bipolar disorder. *PLoS medicine* 2:999.
- Yamada RX, Matsuki N, Ikegaya Y (2006) Nitric oxide/cyclic guanosine monophosphate-mediated growth cone collapse of dentate granule cells. *Neuroreport* 17:661-665.
- Yamazaki M, Chiba K, Mohri T, Hatanaka H (2004) Cyclic GMP-dependent neurite outgrowth by genipin and nerve growth factor in PC12h cells. *European journal of pharmacology* 488:35-43.

- Yang DD, Kuan C-Y, Whitmarsh AJ, Rinón M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* 389:865-870.
- Yang X, Sun P, Qin H, Si P, Sun X, Zhang C (2012) Involvement of MAPK pathways in NMDA-induced apoptosis of rat cortical neurons. *Sheng li xue bao:[Acta physiologica Sinica]* 64:609-616.
- Yun HY, Gonzalez-Zulueta M, Dawson VL, Dawson TM (1998) Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. *Proceedings of the National Academy of Sciences of the United States of America* 95:5773-5778.
- Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres Ba (2012) Genomic analysis of reactive astrogliosis. *The Journal of neuroscience* 32:6391-6410.
- Zarate CA, Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, Charney DS, Manji HK (2006) A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Archives of general psychiatry* 63:856-864.
- Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. *Cell* 132:645-660.
- Zhao Y, Rempe DA (2010) Targeting Astrocytes for Stroke Therapy. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics* 7:439-451.
- Zhao Y, Spigolon G, Bonny C, Culman J, Vercelli A, Herdegen T (2012) The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. *Molecular and Cellular Neuroscience* 49:300-310.
- Zhou H-H, Tang Y, Zhang X-Y, Luo C-X, Gao L-Y, Wu H-Y, Chang L, Zhu D-Y (2015) Delayed Administration of Tat-HA-NR2B9c Promotes Recovery After Stroke in Rats. *Stroke* 46:1352-1358.
- Zhou L, Li F, Xu H-B, Luo C-X, Wu H-Y, Zhu M-M, Lu W, Ji X, Zhou Q-G, Zhu D-Y (2010) Treatment of cerebral ischemia by disrupting ischemia-induced interaction of nNOS with PSD-95. *Nature medicine* 16:1439-1443.
- Zhu L-J, Li T-Y, Luo C-X, Jiang N, Chang L, Lin Y-H, Zhou H-H, Chen C, Zhang Y, Lu W (2014) CAPON-nNOS coupling can serve as a target for developing new anxiolytics. *Nature medicine* 20:1050-1054.
- Zhu W, Zhang S-H, Feng B, Zhong K, Yang L-X, Sun H-L, Zhang S-P, Xu Y, Wang C-Y, Pan X-H (2012) Reactive astrocytes contribute to increased epileptic susceptibility induced by subthreshold dose of pilocarpine. *Epilepsy & Behavior* 25:426-430.
- Zhu Z-H, Yang R, Fu X, Wang Y-Q, Wu G-C (2006) Astrocyte-conditioned medium protecting hippocampal neurons in primary cultures against corticosterone-induced damages via PI3-K/Akt signal pathway. *Brain research* 1114:1-10.
- Zhuang Z-Y, Wen Y-R, Zhang D-R, Borsello T, Bonny C, Strichartz GR, Decosterd I, Ji R-R (2006) A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *The Journal of neuroscience* 26:3551-3560.

Appendix: Publications and Presentations

Publications

- Doucet M, O'Toole E, Connor T, Harkin A (2015) Small-molecule inhibitors at the PSD-95/nNOS interface protect against glutamate-induced neuronal atrophy in primary cortical neurons. *Neuroscience* 301:421-438.
- O'Toole E, Doucet M, Sherwin E, Harkin A (2015) Novel targets in the glutamate and nitric oxide neurotransmitter systems for the treatment of depression. *Book chapter for inclusion in Systems Neuroscience in Depression, published by Elsevier. Accepted; In press*
- O'Toole E and Harkin A (2015) Small-molecule inhibitors of the PSD-95/nNOS interface protect against neuronal atrophy associated with astrocytic ablation. *Neuroscience. Prepared for submission*

Conference proceedings

- O'Toole E, McIntosh, A, Doucet, M, Harkin, A. Small-molecule inhibitors at the PSD-95/nNOS interface protect against glutamate-induced neuronal atrophy in primary cortical neurons. *Poster presentation presented by Dr Allison McIntosh at the British Association for Psychopharmacology (BAP) conference, Bristol, UK, July 2015*
- O'Toole E, Harkin, A. Astrocytic dysfunction induced by L-alpha aminoadipic acid (L-AAA) reduces measures of neuronal complexity; rescue by NMDA receptor antagonists. *Poster presentation - International Society of Neuroimmunology Conference, Mainz, Germany, November 2014*
- O'Toole E, Doucet, M, Harkin, A. Small-molecule inhibitors at the PSD-95/nNOS interface protect against glutamate-induced neuronal atrophy in primary cortical neurons. *Poster presentation at the Royal Academy of Medicine in Ireland section of Biomedical Sciences Annual Meeting, University College Dublin, Ireland, June 2014.*
- O'Toole E, Doucet, M, Harkin, A. Astrocytic ablation by L-alpha aminoadipic acid (L-AAA) reduces measures of neuronal complexity *in vitro*; Rescue by Gliotrophins? *Poster presentation at the Joint Biochemical Society and British Neuroscience Association, Astrocytes in Health and Disease Conference, University College London, April 2014*
- O'Toole E, Doucet, M, Harkin, A. Astrocytic dysfunction induced by L-alpha aminoadipic acid (L-AAA) reduces measures of neuronal complexity. *Poster presentation at the All Ireland Schools of Pharmacy Conference, Trinity College Dublin, April 2014*
- O'Toole E, Doucet, M, Harkin, A. Astrocytic ablation by L-alpha aminoadipic acid (L-AAA) reduces measures of neuronal complexity *in vitro*. *Poster presentation at the Neuroscience Ireland / Irish Area Section Biochemical Society Joint Meeting, University College Cork, Ireland, September 2013*