

**Targeting the NMDA receptor/NO signalling
pathway following L- α amino adipic acid-induced
astrocytic impairment and associated changes in the
structural plasticity of neurons**



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Doctor of Philosophy

By

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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: 3.1, 3.2, 3.4, 3.5, 3.7, 3.8) which were conducted in conjunction with Dr. Eileen O'Toole and Kate O'Reilly.

Eileen O'Toole performed all the work *in vitro* using Ketamine, TRIM and IC87201 and Kate O'Reilly helped with the cell culture and imaging.

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Jennifer David

September 2017

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Summary

This investigation firstly aims to characterise the impact of astrocytic dysfunction on neurons as there is a decrease of the number of astrocytes in the brain of depressed patients. In addition, healthy astrocytes play a role of trophic support whereas when dysfunctional, they can impair neuronal complexity and thus affect neuronal networks. In this study, we were firstly interested to assess *in vitro* the effects of the astrocytic toxin L- α amino adipic acid (L-AAA), a glutamate analogue inducing astrocytic dysfunction and affecting neurite outgrowth and synapse number in primary cortical neurons. Treatment with conditioned media (CM) derived from healthy astrocytes increased neurite outgrowth in immature neurons and synapse number in mature neurons. In contrast, CM derived from L-AAA-treated astrocytes impaired neurite outgrowth in immature neurons and reduced spine density and synapse formation in mature neurons. In addition, L-AAA provoked a reduction in the astrocytic markers glial fibrillary acid protein (GFAP) and the glutamate aspartate transporter (GLAST) and resulted in altered mitochondrial respiration in astrocytes.

The second part of this project focused on the translation from *in vitro* experiments to an animal model of L-AAA-induced astrocytic dysfunction. Testing L-AAA-induced astrocytic dysfunction in mice serves to scale this work by assessing the effect of astrocytic dysfunction on behaviour, neuronal complexity and dendritic spine density in the prelimbic cortex (PLC) *in vivo*. We decided to focus on the prefrontal cortex (PFC) as the size, activation and glutamate concentrations in this area have been shown to be impaired in the brain of depressed patients. In addition, the PLC is a subsection of the PFC involved in the adaptation to environmental stimuli which has been targeted in other studies using L-AAA administration to induce depressive-like behaviours. Following L-AAA delivery to the PLC, transient depressive-like behaviour in the forced swim test (FST) and reduced GFAP immunoreactivity in the PLC were evident over a 72 h post-administration period leading to a full recovery over 7 days. Moreover, L-AAA decreased the stable and mature mushroom-shaped spine variety 72 h after administration with no effect on the number of primary neurites in the PLC.

Finally the effect of targeting the neuronal NMDA-R/NO signalling pathway is assessed as a strategy to prevent neuronal atrophic effects following L-AAA-induced astrocytic dysfunction. Co-treatment of neurons with the NMDA-R antagonist ketamine, as well as the nNOS inhibitor TRIM and the PSD-95/nNOS inhibitors IC87201 and ZL006 partially

protected against reduced neurite outgrowth and synapse formation induced by CM from L-AAA treated astrocytes. In mice, results demonstrate that the PSD-95/nNOS inhibitor (ZL006) had no effect on dendritic spine density or the number of primary neurites. Exposure to the FST reduced thin spine density on apical dendrites with a decrease in thin and mushroom spine density on basal dendrites. ZL006 was able to attenuate FST-induced decreased spine density 24 h after administration. ZL006 and TRIM were also able to block depressive-like behaviour observed in the FST.

Interestingly, FST combined with L-AAA drove the changes in the opposite direction and increased dendritic spine density. ZL006 response reduced thin spine density on basal dendrites and produced an antidepressant-related response in the FST following L-AAA administration.

The results highlight the importance of astrocytes, in particular the consequences of astrocytic impairment on neuronal complexity and synapse formation, and support the hypothesis that selectively targeting the PSD-95/nNOS interaction downstream of the NMDA-R prevents neuronal atrophic effects and potential synapse loss.

Table of contents

DECLARATION	I
ACKNOWLEDGEMENTS	II
SUMMARY	III
LIST OF FIGURES.....	XIII
LIST OF ABBREVIATIONS.....	XVII
CHAPTER 1 INTRODUCTION.....	1
1. NEURONAL PLASTICITY.....	2
1.1. Neurite formation	2
2. THE CYTOSKELETON AND GROWTH CONE	3
2.1. Axon outgrowth	4
2.2. Dendritogenesis	5
2.3. Spines.....	6
2.3.1. Spine formation	6
2.3.2. Spine categorisation	7
2.4. Synaptogenesis	8
3. FACTORS MEDIATING NEURITE OUTGROWTH, DENDRITOGENESIS, DENDRITIC SPINE FORMATION AND SYNAPTOGENESIS ...	10
3.1. Guidance cues for outgrowth.....	10
3.2. Neurotrophic factors.....	12
3.2.1. Neurotrophic factors and neurite outgrowth	13
3.2.2. Neurotrophic factors, spines and synaptogenesis	14
3.3. Glutamate.....	15
3.3.1. Glutamate and neurite outgrowth.....	15
3.3.2. Glutamate, spines and synaptogenesis	16
3.3.3. The glutamate NMDA-R subtype and synaptic plasticity	16
3.3.4. Glutamate mediated NO signalling affects neuronal outgrowth	17
4. NEURONAL ATROPHY IN BRAIN DISORDERS.....	19
4.1. Neurodevelopmental, genetic disorders.....	20
4.2. Neurodegenerative disorders.....	21
4.3. Neuropsychiatric disorders	21
5. MAJOR DEPRESSIVE DISORDER	22
5.1. Depression	22
5.2. Volumetric and structural brain alterations in depression	22
5.3. Functional brain alterations in depression.....	23

5.4.	<i>Animal models of depression</i>	24
5.5.	<i>Effect of stress on neuronal plasticity</i>	25
5.5.1.	<i>Effect of stress on neuronal complexity</i>	25
5.5.2.	<i>Effect of stress on dendritic spines</i>	28
6.	NEW DIRECTIONS FOR THE TREATMENT OF DEPRESSION	32
6.1.	<i>Current pharmacological treatments</i>	32
6.2.	<i>Non pharmacological treatments of depression</i>	34
6.3.	<i>Neurobiology of depression</i>	35
6.3.1.	<i>Mono-amine hypothesis</i>	35
6.3.2.	<i>Glutamatergic hypothesis</i>	35
6.4.	<i>Targeting the glutamatergic system to develop novel therapeutics</i>	36
6.4.1.	<i>Glutamatergic receptors</i>	36
6.4.1.1.	Metabotropic glutamate receptors	38
6.4.1.2.	Ionotropic receptors	38
6.4.1.2.1.	AMPA receptors	38
6.4.1.2.2.	Kainate receptors	39
6.4.1.2.3.	NMDA receptors	39
6.4.2.	<i>NMDA-R antagonists</i>	40
6.4.3.	<i>PSD-95/nNOS modulators</i>	43
6.4.3.1.	PSD-95	43
6.4.3.2.	NO signalling	44
6.4.3.3.	nNOS inhibitors	45
6.4.3.4.	Inhibition of PSD-95/nNOS	46
6.5.	<i>Effect of antidepressant treatment on neuronal plasticity</i>	48
7.	ASTROGLIA AND NEURONAL INTERACTIONS	52
7.1.	<i>Glial cells</i>	52
7.2.	<i>Gliogenesis</i>	55
7.3.	<i>Trophic support of astrocytes</i>	56
7.4.	<i>Dysfunctional astroglia/neuronal interaction</i>	57
7.5.	<i>Evidence of astrocytic abnormalities in depression</i>	59
7.6.	<i>Model of astroglial toxicity</i>	61
7.6.1.	<i>In vitro model of astroglial toxicity</i>	61
7.6.2.	<i>Astrocytic disruption, a new experimental model of depression</i>	62
8.	AIMS AND OBJECTIVES	64
CHAPTER 2 MATERIAL AND METHODS		66
I.	MATERIALS	67
1.	GENERAL CONSUMABLES AND EQUIPMENT	67
1.1.	<i>Consumables</i>	67

1.2.	<i>General equipment</i>	67
2.	IN VITRO STUDIES	68
2.1.	<i>Animals</i>	68
2.2.	<i>Biochemical assays</i>	68
2.3.	<i>Experimental treatments</i>	69
2.4.	<i>Molecular reagents</i>	70
2.5.	<i>Primary and secondary antibodies for immunocytochemistry</i>	70
2.5.1.	<i>Primary antibodies</i>	70
2.5.2.	<i>Secondary antibodies</i>	70
3.	IN VIVO STUDIES	71
3.1.	<i>Animals</i>	71
3.2.	<i>Chemicals and reagents</i>	71
3.3.	<i>Equipment and apparatus for surgical procedures and behavioural testing</i>	72
3.4.	<i>Animal care</i>	72
3.5.	<i>Experimental treatments</i>	72
3.6.	<i>Primary and secondary antibodies for immunohistochemistry</i>	72
3.6.1.	<i>Primary antibodies</i>	72
3.6.2.	<i>Secondary antibodies</i>	73
I.	METHODS	73
1.	IN VITRO EXPERIMENTS	73
1.1.	<i>Preparation</i>	73
1.1.1.	<i>Aseptic techniques</i>	73
1.1.2.	<i>Preparation of culture media</i>	74
1.1.2.1.	<i>Dulbecco's modified Eagle's medium</i>	74
1.1.2.2.	<i>Neurobasal A medium</i>	74
1.1.2.3.	<i>Phosphate buffered saline</i>	74
1.1.3.	<i>Drugs preparation</i>	74
1.2.	<i>Preparation and culture of primary glial cells</i>	75
1.2.1.	<i>Collection of primary cortical mixed glial cells</i>	75
1.2.2.	<i>Astrocyte isolation and re-plating</i>	75
1.2.3.	<i>Astrocyte treatments for CM</i>	76
1.3.	<i>Preparation and culture of primary neurons</i>	77
1.3.1.	<i>Preparation of coverslips</i>	77
1.3.2.	<i>Preparation of primary cortical neuronal cultures</i>	77
1.3.3.	<i>Neuronal cell counting and plating</i>	77
1.4.	<i>RNA Analysis</i>	78
1.4.1.	<i>Harvesting cells for mRNA analysis</i>	78
1.4.2.	<i>RNA Extraction</i>	78

1.4.3.	<i>RNA Quantification and Equalisation</i>	79
1.4.4.	<i>cDNA Synthesis</i>	79
1.4.5.	<i>Real-time PCR (RT-PCR)</i>	79
1.5.	<i>Cell Viability Resazurin (Alamar Blue®) Assay</i>	81
1.6.	<i>Immunocytochemistry</i>	81
1.6.1.	<i>Fluorescent immunocytochemistry for Sholl analysis</i>	81
1.6.2.	<i>Fluorescent immunocytochemistry for the quantification of co-localised puncta</i>	82
1.7.	<i>Image analysis</i>	82
1.7.1.	<i>Sholl analysis</i>	82
1.7.2.	<i>Synaptogenesis analysis for the quantification of co-localised puncta (PSD-95/Synaptophysin)</i>	84
1.8.	<i>GFP transfection</i>	85
1.9.	<i>Glycolysis and mitochondrial respiration measures in astrocytes</i>	86
1.9.1.	<i>Astrocyte preparation and treatment</i>	86
1.9.2.	<i>Mitochondrial respiration and glycolysis measurement</i>	87
1.9.2.1.	<i>Seahorse plate preparation</i>	87
1.9.2.2.	<i>Measurement of OCR using Extracellular Flux Analyser</i>	88
1.9.2.3.	<i>Measurement of ECAR using Extracellular Flux Analyser</i>	89
1.9.3.	<i>Determination of protein quantification</i>	90
2.	IN VIVO EXPERIMENTS	90
2.1.	<i>Animals</i>	90
2.2.	<i>Drug preparations</i>	90
2.2.1.	<i>Intraperitoneal administration</i>	90
2.2.2.	<i>Intra-cortical administration</i>	91
2.3.	<i>Surgery</i>	91
2.3.1.	<i>Aseptic surgical technique</i>	91
2.3.2.	<i>L-AAA administration in the PLC</i>	91
2.3.3.	<i>L-AAA administration in the DG</i>	92
2.3.4.	<i>Evans blue injection</i>	92
2.4.	<i>Behavioural tests</i>	93
2.4.1.	<i>Saccharin preference</i>	93
2.4.2.	<i>Splash test</i>	94
2.4.3.	<i>Open-field test</i>	94
2.4.4.	<i>Tail suspension test</i>	94
2.4.5.	<i>Forced swim test</i>	95
2.5.	<i>Post mortem immunohistochemical analysis</i>	96
2.5.1.	<i>Perfusion</i>	96
2.5.2.	<i>Immunohistochemistry</i>	96
2.5.2.1.	<i>Glial fibrillary acid protein</i>	96

2.5.2.2.	Calcium-binding protein B	97
2.5.2.3.	Neuronal nuclear antigen	97
2.5.2.4.	Co-staining of Doublecortin and GFAP	97
2.5.2.5.	Ionized calcium binding adaptor molecule 1	99
2.6.	<i>Image analysis</i>	99
2.6.1.	<i>GFAP quantitative analysis of cells of interest</i>	99
2.6.2.	<i>S100b quantitative analysis of cells of interest</i>	101
2.6.3.	<i>NeuN analysis of cells of interest</i>	101
2.6.4.	<i>DCX/GFAP quantitative analysis of cells of interest</i>	102
2.6.5.	<i>Iba1 quantitative analysis of cells of interest</i>	103
2.7.	<i>Enhanced golgi-cox staining system for dendrites/spine labelling</i>	104
2.7.1.	<i>Golgi-cox staining</i>	105
2.7.2.	<i>Golgi-cox spine and primary neurite analysis</i>	105
2.8.	<i>Statistical analysis</i>	106

CHAPTER 3 INHIBITORS OF THE NMDA-R/NO SIGNALLING PATHWAY RESCUE NEURONAL ATROPHY AND SYNAPSE LOSS PROVOKED BY L-AAA-TREATED ASTROCYTES.....107

1.	INTRODUCTION	108
2.	AIMS	111
3.	STUDY DESIGN	112
3.1.	<i>Effects of astrocyte CM on neurite outgrowth</i>	112
3.2.	<i>Effects of direct application of L-AAA and astrocyte L-AAA-CM on neurite outgrowth</i>	112
3.3.	<i>Effect of astrocyte L-AAA CM on dendritic spine density in GFP transfected hippocampal neurons</i> 113	
3.4.	<i>Effects of L-AAA on viability of cortical primary neuronal and glial cultures</i>	113
3.5.	<i>Effects of L-AAA on mRNA expression of GFAP and GLAST in astrocytes</i>	114
3.6.	<i>Effects of L-AAA on astrocytic mitochondrial respiration and glycolysis</i>	114
3.7.	<i>Effects of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reductions in neuronal complexity</i>	114
3.8.	<i>Effects of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reductions in the co-localised expression of synaptophysin and PSD-95</i>	115
4.	STATISTICAL ANALYSIS	115
5.	RESULTS	116
5.1.	<i>Effect of 24 h exposure to astrocytic CM on neurite outgrowth</i>	116
5.2.	<i>Effect of 24 h exposure to astrocytic L-AAA-CM on neurite outgrowth</i>	117
5.3.	<i>Effect of 48 h of direct application of L-AAA on neuronal complexity and viability of astrocytes and neurons</i>	119
5.4.	<i>Effect of L-AAA-treated astrocytic CM on spine density in primary hippocampal neurons</i>	122

5.5.	<i>Effect of direct application of L-AAA on mRNA expression of astrocytic markers, OCR and ECAR after 24 h exposure</i>	124
5.6.	<i>Effect of inhibitors of NMDA-R/NO signalling on astrocytic L-AAA CM-induced reduction in neurite outgrowth.</i>	126
5.7.	<i>Effect of inhibitors of NMDA-R/NO signalling on astrocytic L-AAA CM-induced reduction in synapse formation.</i>	128
6.	DISCUSSION	135
6.1.	<i>Effect of astrocytes CM on neurite outgrowth and synapse formation</i>	135
6.2.	<i>Effect of direct application of L-AAA on viability of neurons and astrocytes</i>	136
6.3.	<i>Effect of direct application of L-AAA on mRNA astrocytic markers OCR and ECAR</i>	136
6.4.	<i>Effect of CM from L-AAA-treated astrocytes on neurite outgrowth, synapse formation and dendritic spine density</i>	138
6.5.	<i>Effect of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reduction in neurite outgrowth and synaptogenesis.</i>	139
6.6.	<i>Conclusions</i>	141

CHAPTER 4 DELIVERY OF L-AAA INTO THE PRE-LIMBIC CORTEX (PLC) REDUCES GFAP AND S100B

IMMUNOREACTIVITY AND PROVOKES DEPRESSIVE-LIKE BEHAVIOURS IN MICE 142

1.	INTRODUCTION	143
2.	AIMS	146
3.	STUDY DESIGN	147
3.1.	<i>Surgery</i>	147
3.2.	<i>Behaviour</i>	147
3.3.	<i>Post-mortem analysis</i>	149
4.	STATISTICAL ANALYSIS	149
5.	RESULTS	150
5.1.	<i>Behaviour</i>	150
5.1.1.	<i>Effect of intra-cortical L-AAA administration on bodyweight</i>	150
5.1.2.	<i>Effect of two consecutive daily doses of L-AAA in behavioural tasks.</i>	150
5.1.3.	<i>Effect of a single dose of L-AAA in behavioural tasks</i>	150
5.2.	<i>Post-mortem analysis</i>	153
5.2.1.	<i>Effect of two consecutive daily doses of L-AAA on GFAP, IBA1 and NeuN immunoreactivity in the PLC</i>	153
5.2.2.	<i>Effect of a single dose of L-AAA on GFAP, S100b and NeuN immunoreactivity in the PLC</i>	153
5.3.	<i>Effect of surgery and anaesthesia</i>	156
5.3.1.	<i>Effect of surgery and anaesthesia on bodyweight</i>	156
5.3.2.	<i>Effect of surgery and anaesthesia in behavioural tasks</i>	156
5.3.3.	<i>Effect of surgery and anaesthesia on GFAP and Iba1 immunoreactivity in the PLC</i>	157

6.	DISCUSSION	160
6.1.	<i>Effect of L-AAA in behavioural tasks</i>	160
6.2.	<i>Effect of L-AAA on GFAP, S100b, Iba1 and NeuN immuno-reactivity</i>	161
6.3.	<i>Effect of surgery and anaesthesia in behavioural tasks</i>	162
6.4.	<i>Effect of surgery and anaesthesia on GFAP and Iba1 immunoreactivity</i>	163
6.5.	<i>Conclusion</i>	164
7.	APPENDIX	165
7.1.	<i>Qualitative analysis of the field of distribution of Evan's blue dye following intracortical bolus injection</i> 165	
7.2.	<i>Effect of two doses of L-AAA into the DG on GFAP and DCX immunoreactivity</i>	165

CHAPTER 5 ANTIDEPRESSANT-LIKE PROPERTIES OF ZL006 AND ASSOCIATED CHANGES IN DENDRITIC SPINE DENSITY FOLLOWING DELIVERY OF THE ASTROCYTIC TOXIN L-AAA TO THE PLC IN MICE 167

1.	INTRODUCTION	168
2.	AIMS AND OBJECTIVES	171
3.	METHODS	172
3.1.	<i>Drug administration</i>	172
3.2.	<i>Stereotactic delivery of L-AAA in the PLC</i>	172
3.3.	<i>Behaviour in the mouse FST</i>	172
3.4.	<i>Golgi-cox staining of neurites and dendritic spines</i>	172
4.	STUDY DESIGN	173
4.1.	<i>Effect of ZL006 on dendritic spine density and number of primary neurites</i>	173
4.2.	<i>Effect of FST on dendritic spine density and number of primary neurites</i>	173
4.3.	<i>Effect of ZL006 and TRIM in the mouse FST and associated changes in dendritic spine density and number of primary neurites</i>	174
4.4.	<i>Effect of stereotactic delivery of L-AAA to the PLC on dendritic spine density and number of primary neurites</i>	174
4.5.	<i>Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity</i>	175
5.	STATISTICAL ANALYSIS	175
6.	RESULTS	176
6.1.	<i>Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC in mice</i> .	176
6.2.	<i>Effect of FST exposure on dendritic spine density and number of primary neurites in the PLC in mice</i>	178
6.3.	<i>Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC following FST exposure in mice</i>	180
6.4.	<i>Effect of TRIM on dendritic spine density and number of primary neurites following FST exposure in mice</i>	182

6.5.	<i>L-AAA delivery to the PLC reduces dendritic spine density and the number of primary neurites in the PLC of mice.....</i>	<i>184</i>
6.6.	<i>Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity.....</i>	<i>186</i>
6.6.1.	<i>Effect of FST exposure in L-AAA-treated mice on dendritic spine density and number of primary neurites in the PLC.....</i>	<i>186</i>
6.6.2.	<i>Effect of ZL006 in the FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity.....</i>	<i>188</i>
7.	DISCUSSION	190
7.1.	<i>Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC in the mouse</i>	<i>190</i>
7.2.	<i>Effect of FST exposure on dendritic spine density and number of primary neurites in the PLC in mice</i>	<i>191</i>
7.3.	<i>Effect of ZL006 and TRIM on dendritic spine density and number of primary neurites in the PLC and associated changes in the mouse FST</i>	<i>192</i>
7.4.	<i>L-AAA delivery to the PLC reduces dendritic spine density and the number of primary neurites in the PLC in mice</i>	<i>193</i>
7.5.	<i>Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity.....</i>	<i>194</i>
8.	CONCLUSION	196
	CHAPTER 6 GENERAL DISCUSSION AND FUTURE DIRECTIONS.....	197
1.	CELLULAR/ANIMAL MODELS OF ASTROGLIA DYSFUNCTION	198
2.	NEW ANTIDEPRESSANTS TARGETING THE GLUTAMATERGIC PATHWAY.....	199
3.	INHIBITORS OF NMDA-R/NO SIGNALLING REGULATE DENDRITIC SPINE DENSITY	201
4.	LIMITATIONS AND FUTURE DIRECTIONS	205
	APPENDIX SLICK-H MICE.....	208
1.	SLICK-H TRANSGENIC MICE	209
1.1.	<i>Breeding strategy.....</i>	<i>209</i>
1.2.	<i>Cre/LoxP system.....</i>	<i>209</i>
2.	METHODS.....	211
2.1.	<i>Drug preparations.....</i>	<i>211</i>
2.2.	<i>Stereotactic delivery of L-AAA in the PLC.....</i>	<i>211</i>
2.3.	<i>YFP expression quantification in SLICK-H mice</i>	<i>211</i>
3.	RESULTS	212
3.1.	<i>Assessing neuronal complexity and spine density with SLICK-H mice.....</i>	<i>212</i>
3.2.	<i>Effect of L-AAA on viability of neurons in SLICK-H mice</i>	<i>214</i>
4.	CONCLUSION	215

APPENDIX: PUBLICATIONS AND PRESENTATIONS	216
PUBLICATIONS.....	217
CONFERENCE PROCEEDINGS	217
REFERENCES.....	218

List of figures

FIGURE 1.1: NEURITE FORMATION	3
FIGURE 1.2: THE GROWTH CONE AND NEURITE GUIDANCE.....	4
FIGURE 1.3: SPINE CLASSIFICATION	8
FIGURE 1.4: RHO GTPASES AND NEURITE OUTGROWTH	12
FIGURE 1.5: GLUTAMATE AS A REGULATOR OF OUTGROWTH AND SYNAPSE FORMATION.....	19
FIGURE 1.6: GLUTAMATERGIC SYNAPSE	36
FIGURE 1.7: PROPOSED RAPID MECHANISM OF RAPID ANTIDEPRESSIVE ACTION OF KETAMINE	43
FIGURE 1.8: NMDA-R/PSD-95/NNOS COMPLEX	44
FIGURE 1.9: A SUMMARY OF THE VARIOUS FUNCTIONS OF GLIAL CELLS	53
FIGURE 1.10. DIFFERENT TYPES OF ASTROCYTES.....	54
FIGURE 1.11: GLIOGENESIS.....	55
FIGURE 1.12: SYNTHESIS AND REGULATION OF GLUTAMATE	60
FIGURE 1.13: STRUCTURE OF L-AAA.....	62
FIGURE 2.1: CM TREATMENT AND COLLECTION	76
FIGURE 2.2: SHOLL ANALYSIS.....	84
FIGURE 2.3: COLOCALISATION OF SYNAPTOPHYSIN AND PSD-95	85
FIGURE 2.4: GFP TRANSFECTED NEURONS	86
FIGURE 2.5: ASTROCYTIC CULTURE, ISOLATION AND TREATMENT:.....	87
FIGURE 2.6: PLATE TREATMENT.....	87
FIGURE 2.7: LAYOUT OF THE XF24-WELL CELL CULTURE PLATES, SEAHORSE APPARATUS (BIOSCIENCES).....	88
FIGURE 2.8: MEASURE OF MITOCHONDRIAL RESPIRATION AND GLYCOLYSIS	89
FIGURE 2.9: INJECTION SITE OF L-AAA OR PBS INTO THE PLC.....	92

FIGURE 2.10: SACCHARIN PREFERENCE	93
FIGURE 2.11: TST	95
FIGURE 2.12: FST	95
FIGURE 2.13: NEUROGENESIS	98
FIGURE 2.14: CONFOCAL IMAGING OF GFAP IN THE PLC	100
FIGURE 2.15: CONFOCAL IMAGING OF S100B IN THE PLC	101
FIGURE 2.16: CONFOCAL IMAGING OF NEUN IN THE PLC	102
FIGURE 2.17: CONFOCAL IMAGING OF GFAP AND DCX IN THE DG	103
FIGURE 2.18: IMAGING OF MICROGLIA IN THE PLC	104
FIGURE 2.19: LIGHT MICROSCOPE IMAGING OF GOLGI-COX STAINED NEURONS IN THE PLC.....	106
FIGURE 3.1: EXPERIMENTAL TIMELINE FOR DETERMINATION OF THE EFFECT OF ASTROCYTE CM ON NEURONAL COMPLEXITY.....	112
FIGURE 3.2: EXPERIMENTAL TIMELINE FOR DETERMINATION OF THE EFFECTS OF ASTROCYTE L-AAA- CM OR L-AAA ON NEURONAL COMPLEXITY.....	113
FIGURE 3.3: EXPERIMENTAL TIMELINE FOR DETERMINATION OF THE EFFECTS OF NMDA-R/NO SIGNALLING INHIBITORS ON NEURONAL COMPLEXITY OF NEURONS TREATED WITH ASTROCYTE L-AAA- CM	115
FIGURE 3.4: EXPERIMENTAL TIMELINE FOR DETERMINATION OF THE EFFECTS OF NMDA-R/NO SIGNALLING INHIBITORS ON SYNAPSE FORMATION OF NEURONS TREATED WITH ASTROCYTE L-AAA- CM	115
FIGURE 3.5: EFFECT OF ASTROCYTE CM ON NEURITE OUTGROWTH	117
FIGURE 3.6: EFFECT OF ASTROCYTIC L-AAA CM ON NEURITE OUTGROWTH	119
FIGURE 3.7: EFFECT OF 48 H DIRECT APPLICATION OF L-AAA ON NEURONAL COMPLEXITY AND NEURONAL AND ASTROCYTES VIABILITY.....	121
FIGURE 3.8: EFFECT OF L-AAA-TREATED ASTROCYTIC CM ON DENDRITIC SPINE DENSITY IN PRIMARY HIPPOCAMPAL NEURONS.....	123
FIGURE 3.9.1: EFFECT OF 24 H DIRECT APPLICATION OF L-AAA ON MRNA EXPRESSION OF ASTROCYTIC MARKERS.....	124
FIGURE 3.9.2: EFFECTS OF L-AAA ON GLYCOLYSIS AND OCR IN PRIMARY CORTICAL ASTROCYTES	125
FIGURE 3.10 EFFECTS OF KETAMINE, TRIM, ZL006 AND IC87201 ON L-AAA TREATED ASTROCYTE CM- INDUCED REDUCTION IN NEURAL COMPLEXITY	127
FIGURE 3.12. EFFECTS OF KETAMINE AND TRIM ON THE CO-LOCALISED EXPRESSION OF SYNAPTOPHYSIN AND PSD-95 IN SYNAPTIC PUNCTA.	133

FIGURE 4.1: BEHAVIOURAL TESTING.....	148
FIGURE 4.2: EFFECT OF A SINGLE OR TWO SUCCESSIVE L-AAA DOSES ON BEHAVIOUR.....	152
FIGURE 4.3: INVESTIGATION OF THE EFFECT A SINGLE OR TWO SUCCESSIVE L-AAA DOSES ON IMMUNOREACTIVITY OF GFAP, IBA1 AND NEUN IN THE PLC.....	155
FIGURE 4.4: INVESTIGATION OF THE IMPACT OF SURGERY AND ANAESTHESIA ON BEHAVIOUR	158
FIGURE 4.5: INVESTIGATION OF THE IMPACT OF SURGERY AND ANAESTHESIA ON GFAP AND IBA1 IMMUNOREACTIVITY IN THE PLC.....	159
FIGURE 4.6: REPRESENTATIVE IMAGE OF THE DISTRIBUTION OF EVAN'S BLUE DYE FOLLOWING INTRACORTICAL ADMINISTRATION TO THE MOUSE.....	165
FIGURE 4.7: EFFECT OF TWO DOSES OF L-AAA ON DCX AND GFAP IMMUNOREACTIVITY IN THE DG	166
FIGURE 5.1: EFFECT OF ZL006 ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES	173
FIGURE 5.2: EFFECT OF SWIM STRESS ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES	174
FIGURE 5.3: EFFECT OF ZL006 AND TRIM IN THE MOUSE FST AND ASSOCIATED CHANGES IN DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES.....	174
FIGURE 5.4: EFFECT OF STEREOTACTIC DELIVERY OF L-AAA TO THE PLC ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES	175
FIGURE 5.5: EFFECT OF ZL006 IN THE MOUSE FST AND ASSOCIATED CHANGES IN DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC FOLLOWING L-AAA-INDUCED ASTROCYTIC TOXICITY.....	175
FIGURE 5.6: EFFECT OF ZL006 ADMINISTRATION ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC IN MICE	177
FIGURE 5.7: EFFECT OF FST EXPOSURE ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC IN MICE	179
FIGURE 5.8: EFFECT OF ZL006 ADMINISTRATION ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC FOLLOWING FST EXPOSURE IN MICE	181
FIGURE 5.9: EFFECT OF TRIM ADMINISTRATION ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC FOLLOWING FST EXPOSURE IN MICE	183
FIGURE 5.10: EFFECT OF L-AAA DELIVERY TO THE PLC ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC OF MICE	185
FIGURE 5.11: EFFECT OF FST EXPOSURE IN L-AAA-TREATED MICE ON DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC	187

FIGURE 5.12: EFFECT OF ZL006 IN THE FST AND ASSOCIATED CHANGES IN DENDRITIC SPINE DENSITY AND NUMBER OF PRIMARY NEURITES IN THE PLC FOLLOWING L-AAA-INDUCED ASTROCYTIC TOXICITY	189
FIGURE 6.1: L-AAA-INDUCED ASTROCYTIC TOXICITY AND POTENTIAL MECHANISMS MEDIATING CHANGES IN NEURONAL STRUCTURE AND PLASTICITY	203
FIGURE 6.2: TARGETING NMDA-R/NO SIGNALLING PATHWAY TO PROTECT AGAINST STRESS OR L-AAA-INDUCED NEURONAL ATROPHY	204
FIGURE 7.1: IVF PROTOCOL	209
FIGURE 7.2: THY1-CRE-YFP MOUSE	210
FIGURE 7.3: YFP POSITIVE NEURONS FROM SLICK-H MICE.....	213
FIGURE 7.4: EFFECT OF L-AAA ON THE VIABILITY OF NEURONS	214

List of tables

TABLE 1.1: EFFECT OF STRESS ON NEURONAL COMPLEXITY IN ANIMAL MODELS OF DEPRESSION	27
TABLE 1.2: EFFECT OF STRESS ON DENDRITIC SPINE DENSITY IN ANIMAL MODELS OF DEPRESSION	30
TABLE 1.3. MECHANISMS OF ACTION OF ANTIDEPRESSANTS	34
TABLE 1.4: GLUTAMATE RECEPTORS PROPERTIES	37
TABLE 1.5: EFFECT OF ANTIDEPRESSANT DRUGS ON NEURONAL COMPLEXITY	50
TABLE 1.6: EFFECT OF ANTIDEPRESSANT DRUGS ON SPINE DENSITY	51
TABLE 2.1: PRIMARY ANTIBODIES USED FOR IMMUNOCYTOCHEMISTRY	70
TABLE 2.2: SECONDARY ANTIBODIES USED FOR IMMUNOCYTOCHEMISTRY	70
TABLE 2.3: PRIMARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY	72
TABLE 2.4: SECONDARY ANTIBODIES USED FOR IMMUNOHISTOCHEMISTRY	73
TABLE 2.5: PREPARATION OF STOCK SOLUTIONS	74
TABLE 2.6: LIST OF TAQMAN GENE EXPRESSION ASSAYS USED	80
TABLE 2.7. DRUG PREPARATION FOR IP ADMINISTRATION	91
TABLE 2.8: DRUG PREPARATION FOR INTRA-CORTICAL ADMINISTRATION	91
TABLE 7.1 TAMOXIFEN PREPARATION FOR IP INJECTION	211

List of abbreviations

(Most frequently used abbreviations)

AASST	Attentional set-shifting test
5-HT	5-hydroxytryptamine (serotonin)
AAA	Aminoadipic acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
AMPA-R	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor
Arg	Arginine
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BNST	Bed nucleus of the stria terminalis
BOLD	Blood oxygen level
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
cGKII	Cyclic GMP kinase II
cGMP	Cyclic guanosine monophosphate
CM	Conditioned Media
CNS	Central nervous system
CA1-3	Cornu ammonis 1-3
CREB	cAMP response-element binding protein
CT	Carboxyl terminal
CTD	Carboxyl terminal domain

CUS	Chronic unpredictable stress
Conditioned media	CM
CMV	Chronic mild stress
CRS	Chronic restraint stress
DCX	Doublecortin
DIV	Days in vitro
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
EAAT	Excitatory amino acid transporter
ECAR	Extracellular consumption rates
ECM	Extracellular matrix
ECT	Electroconvulsive therapies
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
eNOS	Endothelial nitric oxide synthase
eYFP	Enhanced yellow fluorescent protein
fMRI	Functional magnetic resonance imaging
FST	Forced swim test
GABA	Gamma-amino butyric acid
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GLAST	Glutamate aspartate transporter
GLT1	Glutamate transporter 1
Gly	Glycine
GS	Glutamine synthetase
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
HPA	Hypothalamic pituitary adrenal
Iba1	Ionized calcium-binding adapter molecule 1

IC87201	(2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl) 4,6dichlorophenol)
iGluRs	Ionotropic glutamate receptors
IL-1	Interleukin-1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IP	Intra-peritoneal
IP3	Inositol trisphosphate
JNK	c-Jun NH2-terminal kinase
kDa	Kilodalton
L-AAA	L-alpha aminoadipic acid
L-Arg	L-arginine
L-NAME	NG-nitro-L-arginine methylester
L-NMMA	NG-monomethyl-L-arginine
L-NNA	NG-nitro-L-arginine
LTD	Long term depression
LTP	Long term potentiation
MAP	microtubule associated protein
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MDD	Major depressive disorder
Mg ²⁺	Magnesium ion
mGluR	Metabotropic glutamate receptor
MPP+	1-methyl-4-phenyl-pyridinium
mM	Millimolar
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MT	Microtubule
mTOR	Mammalian target of rapamycin
mTORc1	Mammalian target of rapamycin complex1
Na ⁺	Sodium ion

NBM	Neurobasal media
NeuN	Neuronal nuclear antigen
NGF	Nerve growth factor
NMDA	N-methyl D aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OCR	Oxygen consumption rates
OF	Open-field
PFA	Paraformaldehyde
mPFC	Medial prefrontal cortex
PBS	Phosphate buffer saline
Polymerase chain reaction	PCR
PFC	Prefrontal cortex
PLC	Prelimbic cortex
PSD	Postsynaptic density
PSD-95	Post-synaptic density 95 kDa
PTEN	Phosphatase tension homolog
RGCs	Retinal ganglion cells
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROCK	RhoA/Rho kinase
ROS	Reactive oxygen species
S100b	S100 calcium-binding protein B
sGC	Soluble guanylate cyclase
SH3	Src homology 3
TBI	Traumatic brain injury
TGF	Transforming growth factor
TNF	Transforming necrosis factor
TRIM	1-[2-(trifluoromethyl)phenyl] imidazole
TSP	Thrombospondin
TST	Tail suspension test
TUNEL	Terminal deoxynucleotidyl

	transferase (TdT) dUTP nick-end labelling
VEGF	Vascular endothelial growth factor
YFP	Yellow fluorescent protein
ZL006	(4-(3,5-dichloro-2-hydroxy- benzylamino)-2-hydroxybenzoic acid)

Chapter 1

Introduction

1. Neuronal plasticity

1.1. Neurite formation

New-born neurons and neural progenitors don't have neurites. The formation of a neurite, the basis for axons and dendrites, starts with a concerted accumulation and organisation of actin and microtubules (MT). During the process of polarisation, neurons develop a single axon and multiple dendrites in a sequence of well-defined developmental stages [for review, ((Lestanova et al., 2016))]. Neurite outgrowth involves the initiation of neurite formation, elongation and guidance of neurites towards their targets and, eventually, synapse formation. *In vivo* observation of neurite formation is difficult due to the experimental requirements; the neurite formation mechanism is more easily observed *in vitro*.

Filopodia are thin, actin-rich plasma-membrane protrusions that function as antennae for cells to probe their environment. There are two models for the initiation of filopodia: the convergent elongation model and the *de novo* nucleation model. The convergent elongation model is based on branched actin filament networks within lamellipodia which are elongated and then bundled into filopodia (Svitkina et al., 2003). The *de novo* nucleation model proposes that filopodia are formed through factors that nucleate actin and elongate actin fibers in a single direction which are then cross-linked into filopodial actin bundles (Vignjevic et al., 2006). Once filopodia are formed, they form neurites. The first model of neurite formation is based on *in vitro*-cultured hippocampal neuron polarisation. It starts (1) by producing actin rich filopodia, spreading around the cell body, (2) the neuron is transformed into a cell, surrounded by a number of short immature processes. (3) One of these processes starts to elongate rapidly and become an axon, (4) the remaining short processes begin to elongate and differentiate into dendrites. This process of polarisation is completed by the maturation of neurites, the formation of dendritic spines and synapses (5) (Dotti et al., 1988; Tahirovic and Bradke, 2009) (**Fig 1.1**). Another mechanism has been listed in the *in vitro* model of cultured cerebellar neurons. Neurite formation differs after (1) the production of the filopodia. (2) Immature neurons first form a single elongated process, (3) followed by another process on the opposite side of the cell body, (4) only one process grows forming branches and axon, (5) while the other process retracts and forms multipledendrites around the cell body (Powell et al., 1997; Tahirovic and Bradke, 2009).

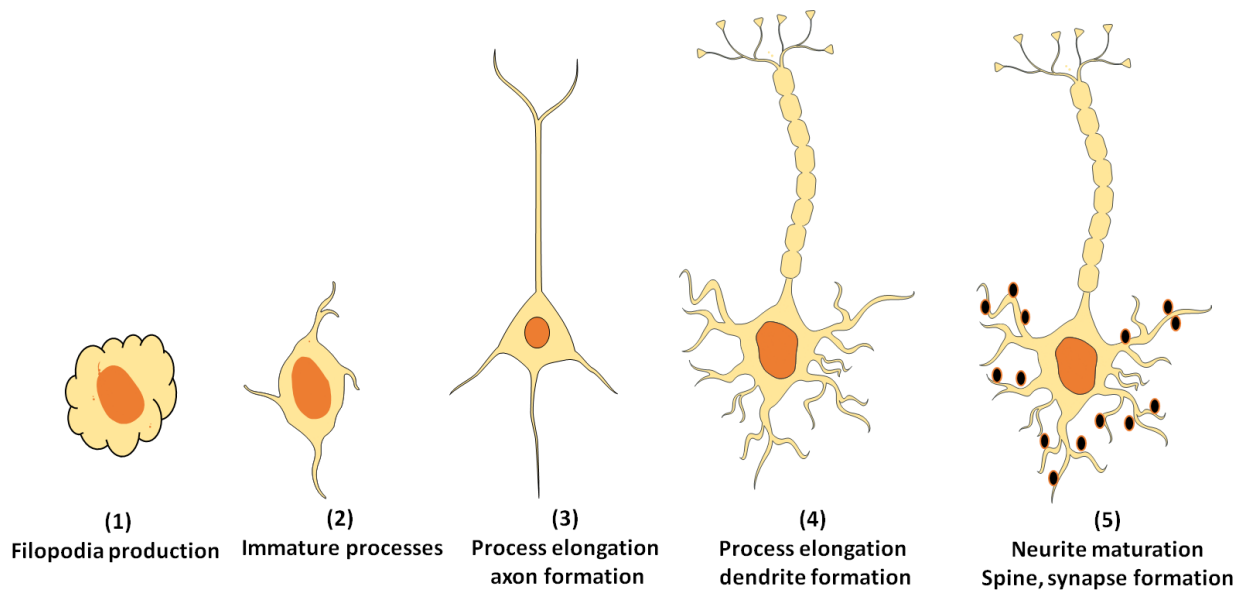


Figure 1.1: Neurite formation

The first model of neurite formation is based on *in vitro*-cultured hippocampal neuron polarisation. It starts (1) by producing actin rich filopodia, spreading around the cell body. (2) The neuron is transformed into a cell, surrounded by a number of short immature processes, (3) one of these processes starts to elongate rapidly and become an axon. (4) The remaining short processes begin to elongate and differentiate into dendrites. This process of polarisation is completed by the maturation of neurites, the formation of dendritic spines and synapses (5).

2. The cytoskeleton and growth cone

The cellular cytoskeleton is divided into three major components: microtubules (MT), microfilament and intermediate filaments. Together, these filamentous proteins determine the cell shape and structure by interacting together and with a multitude of other proteins.

Growth cones, discovered by Cajal in 1888, are small organelles which are the sites of neurite assembly. They are divided into three regions: the peripheral (P) domain, the transitional (T) domain and the central (C) domain. The peripheral domain is made of an actin-based cytoskeleton, mainly composed of finger-like protrusions, filopodia and web-like cytoplasmic veils, lamellipodia (**Fig 1.2**). The central domain is primarily composed of a MT-based cytoskeleton. Although the central region exhibits less plasma membrane dynamics than the periphery, there is substantial molecular motion within this region, including the constant shuttling of organelles and vesicles. The transitional domain is the region located between the central and peripheral domains.

Growth cones are extremely dynamic and can change morphology, extend or retract within seconds to minutes [for review, ((Flynn, 2013))]. Filopodia and lamellipodia play important roles in detecting molecular cues for growth cone adhesion and motility. The actin

cytoskeleton is crucial for determining the shape of the growth cone (Tanaka and Sabry, 1995).

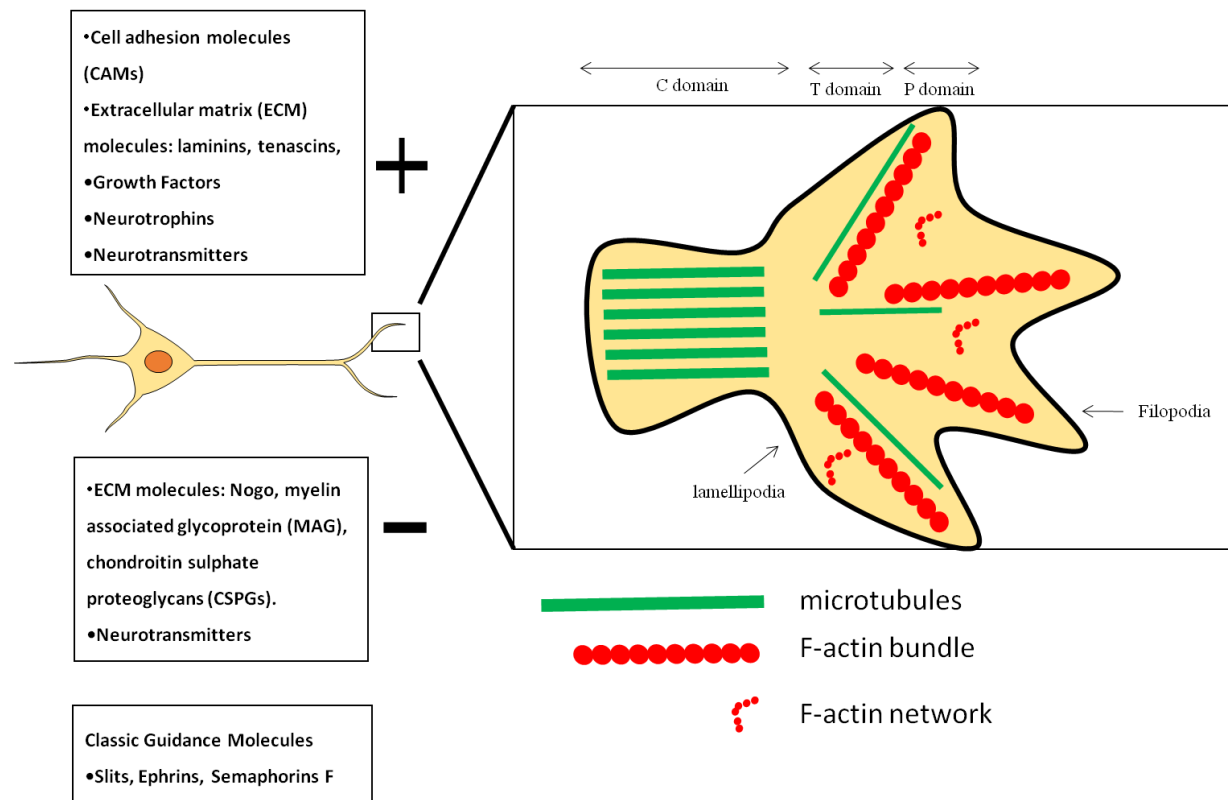


Figure 1.2: The growth cone and neurite guidance

Neurons are guided through their highly responsive growth cones in the extracellular environment. 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 MT. These regions are flanked by the transitional (T) domain which is composed of actin and MT. 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.

2.1. Axon outgrowth

Growth cones destined to become future axons exhibit a significant increase in actin dynamics and instability. Axon outgrowth results from progress through three stages: protrusion, engorgement and consolidation. There is (1) a formation of a protrusion driven by filamentous actin (F-actin) polymerisation at the edges of the growth cone, (2) an engorgement from MT-driven transport of organelles and vesicles, and (3) a consolidation from the contraction and stabilisation of the proximal growth cone into a cylindrical axon shaft [for review, ((Dent et al., 2011))]. In addition to these stages of outgrowth, growth cones also undergo cycles of pausing and retraction involving other cytoskeletal changes. Growth cones assume many shapes and sizes and appear to probe their environment constantly by extending and retracting membrane protrusions (Dent and Gertler, 2003; Lowery and Van Vactor, 2009). Branching can occur through two distinct cellular mechanisms. In principle, a

growth cone can split at the branching point generating two branches. Alternatively, a new branch can emerge from the middle of an axon trunk, a process referred to as interstitial branching. Both of these branching mechanisms depend on proper cytoskeleton dynamics. Branching occurs by local destabilisation of the MT and actin cytoskeleton, followed by an extension of a single filopodium, which is subsequently stabilised by MT invasion (Bastmeyer and O’Leary, 1996; Gallo and Letourneau, 1998; Acebes and Ferrús, 2000; Scott and Luo, 2001).

The growth cone is responsible for the development of both axons and dendrites in neurons. Axons and dendrites have different morphological properties. The axonal arbour sends the information to specific targets via action potential whereas the cell body and dendritic arbour receive the signal. Axons extend for long distances and their terminal branches (growth cones) are converted into pre-synaptic terminals, whereas dendrites extend for a short distance but often branch extensively, giving rise to dendritic trees. Dendrites form post-synaptic specialisations at the end of their branching, creating functional synapses with the pre-synaptic terminals of axons [for review, ((Luo, 2002))].

2.2. Dendritogenesis

A neuron usually forms multiple dendrites but only a single axon. Compared to axons, dendrites possess a plethora of organelles (mitochondria, smooth endoplasmic reticulum and free polyribosome or ribosome). Most of the dendrites receive local inputs, therefore their projections differ from the axons with an average length of 1-2mm versus up to 1m for the axons [for review, ((Penazzi et al., 2016))].

Pyramidal neurons present two different dendritic architectures: the basal and apical dendrites. The apical dendrites arise from the apex of the soma whereas the basal dendrites emerge from the base of the soma (**Fig 2.19**). In contrast, the purkinje cells possess a fan-like dendritic arbour. Apical and basal dendrites present different properties, including size, shape, electrical conduction and responsiveness to neurotrophic factors or guidance molecules (McAllister et al., 1995; Häusser et al., 2000; Arikath, 2012). A recent study showed that polarised growth of the apical dendrite is regulated by cell intrinsic programmes, while regression of basal dendrites requires cues from the extracellular environment in the dentate gyrus (DG) (Wu et al., 2015).

The cytoskeletal requirements of dendritic growth, guidance and branching have not been examined as extensively as those of axons (or neurites in cases of cultured neurons where axonal/dendritic differentiation is not apparent). Dendritic outgrowth is a progressive and stable process of elongation that begins shortly after axonal growth or in some cases after the formation of the first synaptic contacts. There is a switch between axonal and dendrite growth, as neurons start to elongate dendrites when they lose the ability to elongate axons (Goldberg, 2003). The development of dendritic branches is governed by dynamical extension, stabilisation and pruning (Parrish et al., 2007). Taking into account the similarities between axons and dendrites, it is possible that they react in an identical manner for certain aspects of the cytoskeletal basis for growth, guidance and branching.

Indeed, the majority of molecules involved in signalling and guidance regulate in a similar way both dendritic and axonal morphogenesis [for review, ((Scott and Luo, 2001))]. However, certain molecules have differential effects on axons and dendrites, such as the guidance molecule semaphorin 3A which generally promotes dendrite outgrowth while inhibiting axonal outgrowth (Gallo, 2006). The extension and branching of apical and basal dendrites are also differentially regulated by semaphorin 3A (Polleux et al., 2000; Fenstermaker et al., 2004; Tran et al., 2009).

Dendrites and axons differ in growth rates and MT polarity, involving differences in the regulation of cytoskeletal dynamics [for review, ((Craig and Banker, 1994))]. The mechanisms involved in dendritic branching are similar to those observed in axons. They occur either by splitting the growth cone or by interstitial branching and subsequent branch addition from stabilised branches. In addition, observations from hippocampal developing pyramidal neurons (slices) revealed that dendritic shafts constantly extend and retract filopodia. Some of these filopodia are stabilised into new dendrites in early development, whereas in later development these filopodia can form dendritic spines (Dailey and Smith, 1996).

2.3. Spines

2.3.1. Spine formation

Neurons generate small protrusions on their dendritic trees called dendritic spines which are the site of most excitatory synapses in the mammalian brain and are believed to play important functions in learning and memory. Dendritic spine formation is a critical step in dendritic outgrowth, as dendritic spines represent the primary sites of synaptic contact in the brain. The early development of spines shares many characteristics with branching

development. Electron microscopy, myosin decoration, immunohistochemistry and actin-green fluorescent protein (GFP) fusion experiments demonstrated that dendritic spines are highly enriched in actin (Fifková and Delay, 1982; Matus et al., 1982; Landis and Reese, 1983; Fischer et al., 1998). Overall, the main difference between spines and branching points is that spines predominantly contain actin filaments instead of MT filaments. Upon contact with axons, post-synaptic specialisations formed on dendrites create functional synapses with pre-synaptic axonal terminals (Luo, 2002)

There are 3 main theories for spinogenesis [for review, (García-López et al., 2011)]. The Sotelo model proposes that dendritic spines from purkinje neurons emerge from dendritic trees by an autonomous cellular programme, independently of the axonal terminals. The Miller/Peters model claims that dendritic spines from cerebellar neurons are induced by axonal terminals on dendrites by a successive outgrowth through a sessile spine to a pedunculated one. Lastly, the filopodial model proposes that filopodia are able to become dendritic spines which interact with axons. To conclude, in some cases dendritic spines can be formed without the assistance of filopodia. Therefore, filopodia could help facilitate axodendritic synaptic contacts, increasing the dendritic capture volume.

2.3.2. Spine categorisation

It is widely believed that the functional properties of dendritic spines are highly correlated with their morphology. Parameters such as the dimension of spine head and spine neck determine different aspects of dendritic spine function, including the abundance of neurotransmitter receptors, the diffusion of small molecules between spine and shaft, as well as the motility and stability of the spine (Lai and Ip, 2013). Spines are remarkably dynamic, changing size and shape over timescales ranging from seconds to days (De Roo et al., 2008). Filopodia on developing dendrites have been shown to have lifetimes of only minutes maturing into more stable spines that can maintain their morphology for hours (Parnass et al., 2000). Spines present diverse morphologies with long, thin spines known as filopodia maturing into thin, mushroom, stubby or cup-shaped (branches) spines in response to synaptic activity (**Fig 1.3**) (Hering and Sheng, 2001). Each spine has a post-synaptic density (PSD) at its tip that becomes aligned with the pre-synaptic membrane of a target axon terminal. The formation of spines may help to compartmentalise excitatory intracellular activity and the different morphologies may play a role in the regulation of the communication between synapses and dendrites (Hering and Sheng, 2001).

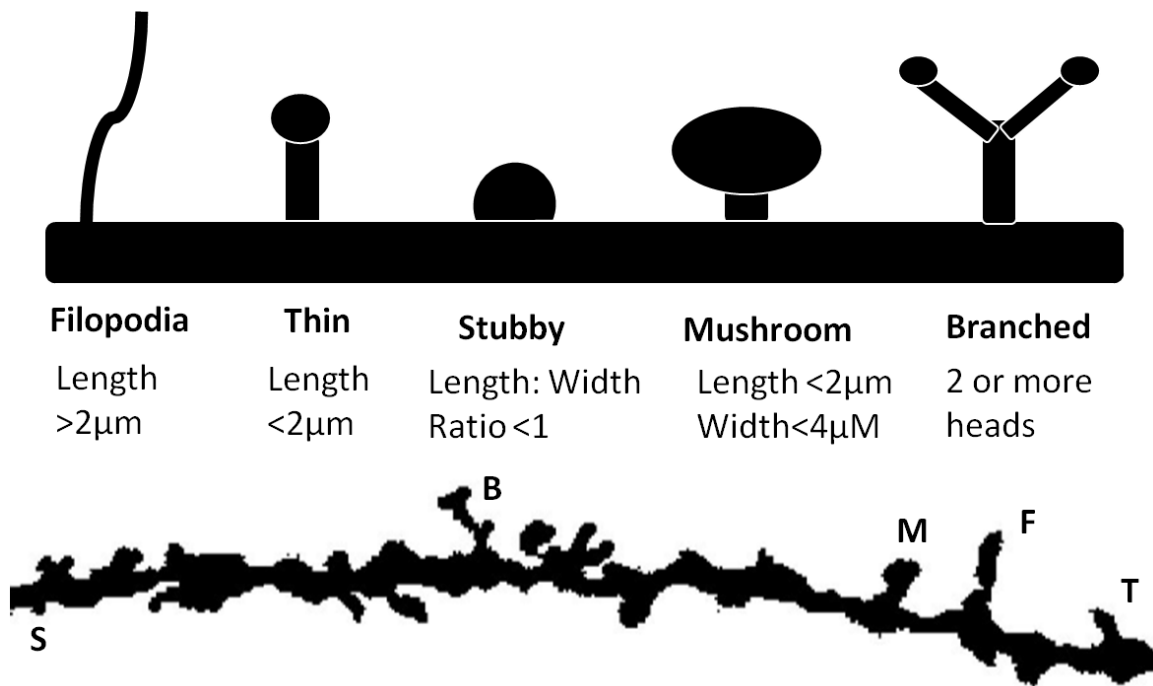


Figure 1.3: Spine classification

Spines present diverse morphologies with long, thin spines known as filopodia (F) maturing into thin (T), mushroom (M), stubby (S) or cup-shaped spines (B) in response to synaptic activity.

2.4. Synaptogenesis

Synaptogenesis is the last stage of processes including neurite initiation, polarity specification, axon guidance, dendritic growth, spine formation and target selection. Synapse formation is a quick process, as synapses can be formed in less than 2h (Cohen-Cory, 2002). Generally, synaptogenesis is defined as a specialised cell-cell adhesion between the pre-synaptic axon of one neuron and the post-synaptic dendrite of another neuron. However, synapses can also be formed at the growth cones of developing neurites and at multiple sites along axonal and dendritic shafts. Most of the synaptic material required for synapse formation is synthesised in the soma of neurons and transported to the synapses by the MT. During this process, the pre-synaptic terminal accumulates vesicles, and the post-synaptic terminal recruits neurotransmitter receptors [for review, (Baldwin and Eroglu, 2017)].

Excitatory synapses are particularly concentrated between contacts with axonal terminals and post-synaptic dendritic spines (Hering and Sheng, 2001; Fiala et al., 2002; Nimchinsky et al., 2002). In contrast, inhibitory synapses on Gamma-amino butyric acid (GABA)ergic neurons are formed between axonal terminals and the dendritic shaft [for review, ((Levinson and El-

Husseini, 2005)]. Neuroligins seem to play a major role in the direction of development into an inhibitory or excitatory synapse [for review, (Jüttner and Rathjen, 2005)]. Neuroligin-1 is primarily found on the post-synaptic side of excitatory synapses whereas neuroligin-2 is expressed predominantly in inhibitory synapses (Song et al., 1999; Graf et al., 2004; Varoqueaux et al., 2004). Despite the controversy about whether excitatory synapses on glutamatergic neurons are initially formed on dendritic filopodia or dendrite shafts, and after maturation formed on spine (Ziv and Smith, 1996; Fiala et al., 1998; Okabe et al., 2001), changes in spine morphogenesis is clearly a critical event in the maturation of glutamatergic synapses [for reviews, (Yuste and Bonhoeffer, 2004; Tada and Sheng, 2006)]. Synaptic maturation consists in an increase in synapses width with an augmentation of pre- and post-synaptic proteins. In cultured neurons, immediately after synapse formation, there is an accumulation at the pre-synaptic site of vesicles containing synaptic proteins triggering neurotransmitter release (Ahmari et al., 2000). Simultaneously in cortical neurons (or later in hippocampal neurons), synaptic components such as the PSD and glutamate receptors accumulate at the post-synaptic site (Washbourne et al., 2002).

The PSD possess scaffolding proteins, such as post-synaptic density 95 kDa (PSD-95) and cytoskeletal proteins. Once a synaptic contact is formed, released neurotransmitters accumulate at the PSD and there is an increase in trafficking of several protein complexes, such as synaptic vesicles, cell adhesion molecules and synaptophysin-containing transport packets in the pre-synaptical terminal (Goldberg, 2003). These proteins accumulate and form the pre-synaptic ‘puncta’, a cluster of synaptic proteins believed to be markers of stable synapses. Accumulation of synaptic puncta also occurs at the post-synaptic neuron. F-actin is highly concentrated in pre- and post- synaptic terminals. F-actin is required for pre-synaptic assembly during the early stages of synaptogenesis. *In vitro*, young hippocampal neurons but not mature neurons showed a reduction in the size and number of synapses after the depolymerisation of F-actin (Zhang and Benson, 2001).

The number of synapses can be decreased by “axonal pruning”, enabling the removal of additional dendritic branches and the activation of intracellular processes which inhibit further dendritic growth (Colón- Ramos, 2009). The strengthening of synapses in adulthood occurs during long-term potentiation (LTP), the major cellular mechanism underlying learning and memory. LTP increases the size of dendritic spines, concurrent with increased

levels of actin filaments and MT entry (Gordon-Weeks and Fournier, 2014). LTP also enhances trafficking of glutamate receptors to the synapse (Cline, 2001).

3. Factors mediating neurite outgrowth, dendritogenesis, dendritic spine formation and synaptogenesis

3.1. Guidance cues for outgrowth

The ability of neurites to navigate through their environment can be controlled by attractive and repulsive cues (**Fig 1.2**), either at the cell surface bound or diffusible molecules forming concentration gradients to guide the growth of axons [for review, ((Tessier-Lavigne and Goodman, 1996)]. Growth cones guided to their targets are often classified into mechanisms of chemo-repulsion, chemo-attraction, contact-dependent repulsion and contact-dependent attraction (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Growth cone turning is mediated by the level of negative or positive cues across a growth cone. The growth cone turns as a result of changes in the actin cytoskeleton, enabling neuritic growth in the presence of an attractive cue, or selective destabilisation of actin cytoskeleton in the presence of a negative cue (Luo, 2002).

Several signalling factors ranging from diffusible gradients of neurotransmitters, growth factors and neurotrophin factors (Goldberg, 2003), to ECM molecules, such as permissive laminins and tenascins (Kamiguchi and Lemmon, 2000), and repulsive Nogo, Myelin associated glycoprotein, chondroitin sulphate and proteoglycans (Snow et al., 1990; Beller and Snow, 2014) direct neurites towards their correct target. In addition, cell adhesion molecules (CAMs) (Williams et al., 1994; Ranscht, 2000; Rigato et al., 2002; Kiryushko et al., 2004; Hansen et al., 2008) or guidance molecules, such as ephrins, netrins, semaphorins and slits as well as various neurotrophins and neurotransmitters (Gallo and Letourneau, 2000) also play a role to direct neurites.

These positive or negative cues initiate signalling cascades activating neuronal proteins which directly impact the actin and MT cytoskeleton (Dent et al., 2011, p. 2). These signals modulate the cytoskeleton by interacting with actin-binding proteins and steering the growth cone. They include the Rho guanosine triphosphatases (GTPases) thought to be responsible for promoting or inhibiting outgrowth (Gomez and Letourneau, 2014). Rho GTPases include members such as RhoA, RAC1 and cell division control protein 42 (Cdc42), and act by linking 8 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). RAC1 also induces synaptic remodeling in stress disorders and depression (Golden et al., 2013; Wang et al., 2018) and is able to regulate fear memory (Gan et al., 2016).

In general, Rac1 and Cdc42 are activated by attractive cues and promote axonal and dendritic outgrowth, while RhoA is activated by repulsive cues and inhibits outgrowth (**Fig 1.4**) (Schwamborn and Püschel, 2004).

RAC activates (1) PAK and subsequently the mitogen-activated protein kinase (MAPK) and (2) the phosphoinositide 3-kinase (PI3K)/Akt. The positive effect on their activation on neurite outgrowth has been extensively studied, particularly in response to neurotrophic factors (Barnes and Polleux, 2009). In contrast, the inhibition of Akt by the Akt-inhibitory phosphatase tension homolog (PTEN) activated by the Rho kinase reduces neurite outgrowth (Stankiewicz and Linseman, 2014; L.-L. Wang et al., 2017). Therefore, a balance between RAC and Rho determines the positive or negative effect on outgrowth (Stankiewicz and Linseman, 2014).

In addition to the Rho GTPases, actin dynamics can also be modulated by non-receptor tyrosine kinases (RTKs) such as focal adhesion kinase and Src family kinases (Gomez and Letourneau, 2014). Changes in cytosolic Ca^{2+} concentration in the growth cone can also regulate the actin cytoskeleton, having a positive or a negative influence on growth cone motility by regulating cyclic adenosine monophosphate (cAMP) levels. Interestingly, in the case of neurotrophins, neurotransmitters and netrins, it was shown that a single growth cone can respond to the same diffusible cue with an attractive or a repulsive response depending on the level of cAMP in the growth cone (Ming et al., 1997; Song et al., 1999). The response of 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).

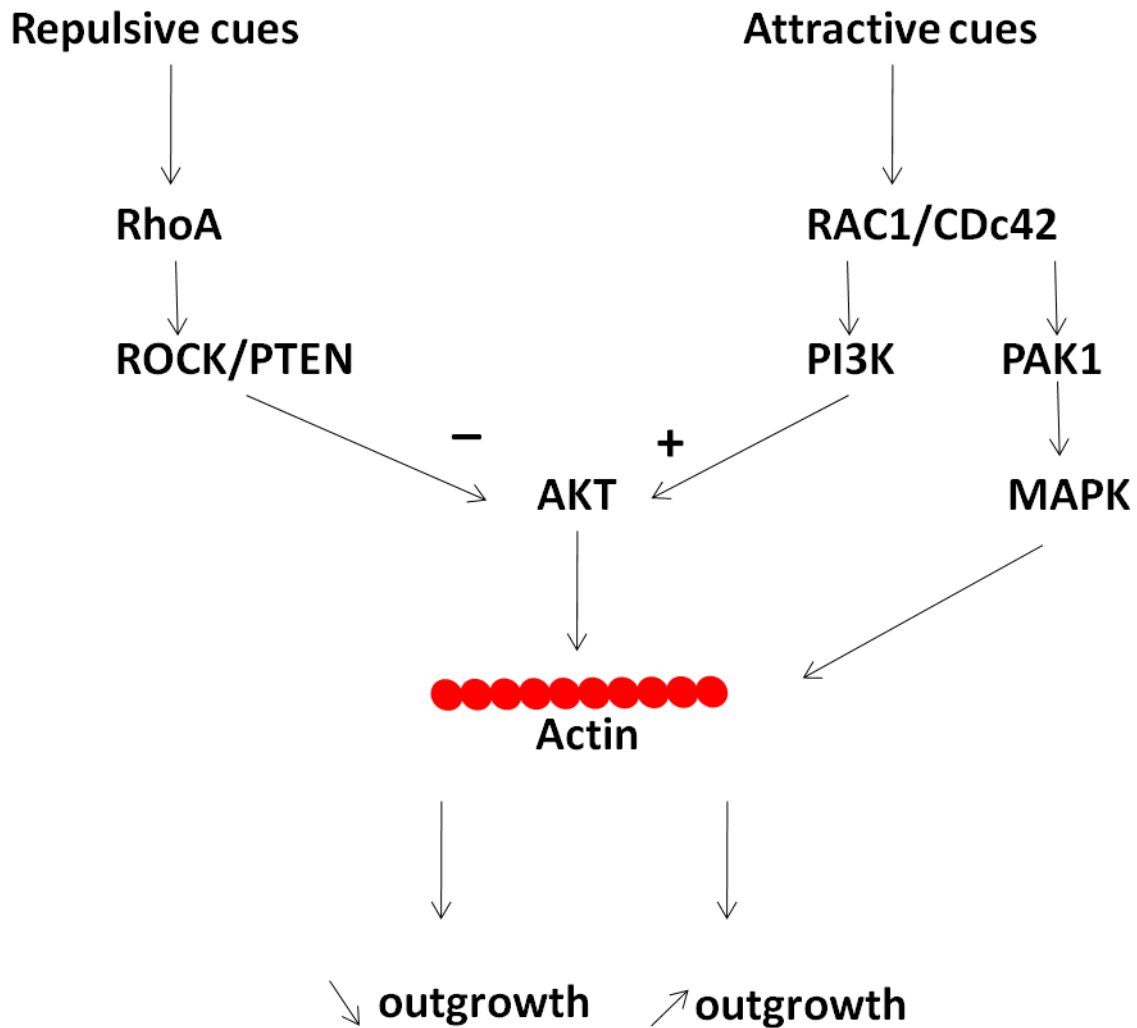


Figure 1.4: Rho GTPases and neurite outgrowth

The activation of Rho GTPases Rac1 and Cdc42 leads to (1) the activation of serine/threonine protein kinase 1 (PAK1) activating MAPK and (2) Phosphoinositide 3-Kinase (PI3K) activating AKT, resulting in cytoskeletal rearrangement that promotes neurite outgrowth. The activation of Rho GTPase RhoA leads to the activation of Rho-associated protein kinase (ROCK)/ Phosphatase and tensin homolog (PTEN) inhibiting AKT and actin-binding proteins, resulting in cytoskeletal changes that inhibit neurite outgrowth.

3.2. Neurotrophic factors

Neurotrophic factors are involved in the growth and survival of immature neurons and the maintenance of mature neurons. They are released by the target cells in order to guide the growth of the developing neurite. They are composed of neurotrophins, the nerve growth factor (NGF), the brain-derived growth factor (BDNF) and the neurotrophins-3 and -4 (NT-3, NT-4), but also the glial-derived neurotrophic factor (GDNF), the fibroblast growth factor (FGF-2), the insulin-like growth factor (IGF-1), the vascular endothelial growth factor (VEGF), the transforming growth factor β (TGF- β), the ciliary neurotrophic factor (CNTF) and the cytokines. All of these have different cell signalling mechanisms although their cellular responses often overlap.

3.2.1. Neurotrophic factors and neurite outgrowth

Interest in the involvement of neurotrophins in activity-dependent synaptic plasticity grew after neurotrophin synthesis was shown to be increased by both seizure activity and sensory stimulation (Thoenen, 1991). They are initially produced as pro-neurotrophins, which must be cleaved to produce their mature forms. Interestingly, pro-neurotrophins and neurotrophins appear to play opposite role in neurite outgrowth (Park and Poo, 2013; Kowiański et al., 2017). Pro-neurotrophins preferentially bind to the p75NTR over the Trk receptors and their binding induces apoptosis. The activation of the p75NTR leads to stimulation of the c-Jun N-H2 terminal kinase (JNK) stream of the MAPK pathway and ceramide signalling (Chao, 2003, p. 200). These pathways are associated with cell death and apoptosis. Research revealed that neurotrophins regulate neuronal functions including cell proliferation and differentiation, axon and dendrite growth, synaptogenesis, synaptic function and plasticity [for review, (Park and Poo, 2013)]. By binding at their respective receptors at the growth cone, neurotrophin factors induce neurite extension (Goldberg, 2003). The activation of the neurotrophic factor receptors induces a cascade activation of the MAPK, PI3K, and STAT3 signalling pathways, contributing to neurite outgrowth (Redmond et al., 2002; Teng and Tang, 2006; Read and Gorman, 2009). Through the activation of these pathways, neurotrophic factors can regulate the activity of Rho GTPases (Cohen- Cory et al., 2010).

In vitro, an extracellular gradient of neurotrophin is able to cause chemotropic turning of axonal growth cones (Song and Poo, 1999). *In vivo*, in developing mouse limb buds, sensory axons can be directed towards beads containing NGF, BDNF, NT-3 or NT-4 (Tucker et al., 2001). Neurotrophin factors also impact dendritic outgrowth (McAllister et al., 1995). Each of the neurotrophin factors induces dendritic outgrowth of neurons in development *in vitro* and *in vivo* (McAllister et al., 1999). BDNF increased dendritic outgrowth in adult-born mouse hippocampal neurons (Wang et al., 2015) and promoted *Xenopus* axonal branching *in vivo* (Cohen-Cory and Fraser, 1995). In addition, dendrites locally release neurotrophins to attract axons. Local secretion of BDNF from the cell body or single dendrites increased growth and branching of nearby dendrites in ferret brain slices (Horch and Katz, 2002).

GDNF was originally discovered for its effects on the survival of dopaminergic neurons (Lin et al., 1993). However, it has also been found to have trophic effects on noradrenergic neurons (Arenas et al., 1995). FGF-2 enhances neurite outgrowth of rat primary hippocampal neurons (Patel and McNamara, 1995). The VEGF-induced activation of VEGFR2 and Rho GTPases stimulates neurite outgrowth in primary cortical neurons (Jin et al., 2006).

Interleukin-6 (IL-6) promotes the neurite outgrowth of primary hippocampal neurons through the activation of the Jak/STAT pathway (Sarder et al., 1996). CNTF promotes the neurite outgrowth of dorsal root ganglion neurons also through the activation of the Jak/STAT pathway, as well as the PI3K pathway (Liu et al., 2014).

3.2.2. Neurotrophic factors, spines and synaptogenesis

Neurotrophic factors also play a role in synapse formation and maturation (Park and Poo, 2013). Neurotrophins induce the formation of new synapses and modulate the structure and function of existing synapses (Cohen-Cory, 2002).

BDNF-induced axonal branching is accompanied by an increase in synapse number in developing neurons (Sanchez et al., 2006), dendritic spines formation and efficiency of synaptic transmission (Kumar et al., 2005; Amaral and Pozzo-Miller, 2007; Gonzalez et al., 2016). In mature hippocampal neurons, BDNF induces neurite outgrowth and the growth of dendritic spines containing PSD-95 (Wang et al., 2015). BDNF also increases the number of spines in rat hippocampus (Tyler and Pozzo-Miller, 2003) and synaptic proteins in the rat cortex (Takei et al., 1997).

Interestingly, BDNF gene delivery was able to reverse synapse loss in a mouse model of Alzheimer's disease (Nagahara et al., 2009). BDNF over-expression led to an increase in synapse and synaptic vesicle number (Aguado et al., 2003) while BDNF, TrkB and TrkC KO mice presented with reduced expression of synaptic proteins, synaptic vesicles and synaptic density (Pozzo-Miller et al., 1999; Otal et al., 2005). In addition, in cultured hippocampal neurons, BDNF promoted the formation of both excitatory and inhibitory synapses, whereas NT-3 only promoted the formation of excitatory synapses (Vicario-Abejón et al., 2002). Applying exogenous BDNF provoked a rapid conversion of non functional contacts between cultured hippocampal neurons into functional synapses, blocked with BDNF disturbance (Shen et al., 2006).

NGF promoted GABAergic synaptogenesis in hippocampal neurons *in vitro* (Salama-Cohen et al., 2006). Other neurotrophic factors can also influence synaptogenesis. GDNF signalling through GFR α 1 increased synapse number in hippocampal neurons (Ledda et al., 2007) and was more recently found to upregulate expression of synaptophysin in enteric neurons (Böttner et al., 2013). FGF-2 has been shown to increase hippocampal synaptogenesis in rats through the activation of the MAPK pathway (Li et al., 2002). VEGF activity is implicated in

the regulation of dendritic spine number and density in mouse adult-born neurons (Licht et al., 2010) and has been found to interact with N-methyl-D aspartate receptors (NMDA-Rs) on newly-formed synapses (Meissirel et al., 2011). IGF-I has been shown to regulate neuronal maturation, affecting axonal and dendritic growth and increasing synapse formation (O’Kusky et al., 2000; Cao et al., 2011). IGF-I over-expression in transgenic mice promoted dendrite growth and synaptogenesis in the DG (O’Kusky et al., 2000; Carlson et al., 2014). Wnt, TGF- β , and tumor necrosis factor α (TNF- α) largely promote synaptogenesis and/or synaptic transmission but also act as negative regulators (Shen and Cowan, 2010; Krieglstein et al., 2011; Koles and Budnik, 2012; Salinas, 2012).

3.3. Glutamate

Glutamate is the major excitatory neurotransmitter in the CNS. Although glutamate is mostly known for its role in synapses in the mature nervous system, it also plays a developmental role by regulating neurogenesis, neurite outgrowth, synaptogenesis and apoptosis (Mattson, 2008). Glutamate is crucial for normal physiological functions of the CNS and stimulates the production of neurotrophic factors such as BDNF, which in turn promotes neurite outgrowth and synaptogenesis. However, excess in glutamatergic stimulation can affect neurons and lead to neuronal atrophy, degeneration and excitotoxicity through excessive Ca^{2+} influx and the activation of Ca^{2+} dependent signalling molecules (Willard and Koochekpour, 2013). Glutamate allows rapid dendritic and cytoskeletal changes via the elevation of Ca^{2+} levels which activate the calcium/calmodulin-dependent protein kinase II (CaMKII), PKC and transcription factors such as cAMP response element-binding protein (CREB) (Miyamoto, 2006; Mattson, 2008). Excitotoxicity results in the production of reactive oxygen species (ROS) and the abnormal activation of proteases and caspases involved in neuronal death.

3.3.1. Glutamate and neurite outgrowth

Glutamate, released from growth cones, can act on the growing dendrites of adjacent cells to alter their outgrowth and promote synaptogenesis (Mattson et al., 1988). Several studies indicate that glutamate at a physiological dose acts as a permissive cue for outgrowth, whereas glutamate excess is able to induce neuronal atrophy (Mattson et al., 1988; Metzger et al., 1998; Esquenazi et al., 2002; Podestá et al., 2014), dendrite regression and spine loss (Monnerie et al., 2003). Glutamate regulates neurite outgrowth by affecting cytoskeletal dynamics in the growth cone and neurite shaft. Glutamate-induced Ca^{2+} influx may cause a rapid local polymerisation of actin to form filopodial extensions of growth cones, while

inhibiting tubulin polymerisation and hence neurite elongation. The sustained elevation of intracellular Ca^{2+} in response to glutamate receptor activation can result in depolymerisation of microfilaments and MT resulting in dendrite outgrowth cessation and regression.

3.3.2. Glutamate, spines and synaptogenesis

Glutamate receptors [Glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-R) and glutamatergic NMDA-R are recruited at the postsynaptic density in less than 1h (Friedman et al., 2000; Washbourne et al., 2002). The depolarisation of the NMDA-R leads to a local Ca^{2+} influx and subsequent activation of intracellular signalling pathways is followed by a progressive recruitment of AMPA-R. The increase in the AMPA-R/NMDA-R ratio at these synapses is associated with the stabilisation of dendritic spines (Shepherd and Huganir, 2007). Immature synapses that contain NMDA-Rs but not AMPA-Rs are termed silent synapses as the recruitment of AMPA-Rs leads to a functional synapse. Post-synaptic AMPA-Rs also regulate the stability of pre-synaptic inputs (Ripley et al., 2011).

Glutamate is able to inhibit dendrite outgrowth of neurons in the entorhinal cortex, and to promote synaptogenesis in hippocampal neurons. Similar roles for glutamate in regulating synaptogenesis in the developing visual system have been reported (Cline and Constantine-Paton, 1990).

AMPA-R and NMDA-R play a key role in LTP. By activating kinases and phosphatases, Ca^{2+} influx mediates the local remodelling of postsynaptic spines. Lower levels of stimulation may result in long-term depression (LTD) of the synapses, whereas sustained overstimulation can cause degeneration of the synapses (Massey and Bashir, 2007).

3.3.3. The glutamate NMDA-R subtype and synaptic plasticity

NMDA-Rs play a pivotal role in synaptic transmission and plasticity (Volianskis et al., 2015). NMDA-Rs form heterotetrameric complexes mainly composed of two GluN1 subunits together with either two GluN2 subunits or a combination of GluN2 and GluN3 subunits (Monyer et al., 1994). At the majority of excitatory synapses, NMDA-R activation is required for LTP or LTD induction. GluN2B subunits undergo more frequent endocytosis than GluN2A. GluN2B enters into recycling endosomes and GluN2A into late endosomes (Lavezzari et al., 2004). This suggests that the activity- or neuromodulatory-dependent regulation of GluN2B may be a quicker or more sensitive way to alter the state of a synapse.

GluN2B subunit is crucial for brain development, synaptic plasticity and neuronal survival (Barria and Malinow, 2002; Thomas et al., 2006; Muñoz et al., 2014). A recent study showed that GluN2B is involved in the induction of LTD and LTP in slices prepared from post-natal 14 (P14) rat hippocampus (France et al., 2017). NMDA-R subunits may influence plasticity with their long carboxy terminal domain (CTD), as these allow for many intracellular interactions and modifications by phosphorylation. There is a high affinity binding between the GluN2B CTD and the catalytic domain of CaMKII (Strack and Colbran, 1998). The activation of CaMKII leads to LTP induction (Foster et al., 2010). Over-expressing GluN2B CTD disrupts the physiological interaction between NMDA-R/CaMKII and impairs LTP in the hippocampus (Zhou et al., 2007). LTP in organotypic hippocampal slices was impaired after expression of a mutant synaptic GluN2B subunit reducing the binding with CaMKII (Barria and Malinow, 2005).

In addition, the GluN2B subunit co-localises with PSD-95 and therefore modulates neuronal nitric oxide synthase (nNOS) upon stimulation (Jay E. Brenman et al., 1996). Following excitotoxic stimuli or ischemia, PSD-95 couples nNOS to GluN2B, enabling the activation of nNOS through Ca^{2+} influx and generating NO (Dawson et al., 1991; Sattler et al., 1999). Interestingly, disrupting GluN2B-PSD-95 interaction prevented PSD-95 from recruiting nNOS to the NMDA-R complex, thereby reducing NO production and NO-mediated neuronal death from ischemia (Aarts et al., 2002). Glutamate up-regulates GluN2B translation through NO, which induces the phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2 α) (Ramos-Fernández et al., 2016).

3.3.4. Glutamate mediated NO signalling affects neuronal outgrowth

It has been shown that glutamate-induced Ca^{2+} mediates cytoskeletal dynamics and filopodial extension (Kuo et al., 2010), which in turn activates the PSD-95/nNOS pathway. Previous research demonstrated the role of PSD-95 and NO in dendritogenesis and synapse formation (Charych et al., 2006). Regarding the formation of NO, nNOS is closely coupled with the Ca^{2+} permeable NMDA-R at the PSD through their mutual PDZ binding motifs (Jay E Brenman et al., 1996). The activation of nNOS is classically Ca^{2+} /calmodulin-dependent. Free cytosolic Ca^{2+} binds to calmodulin and activates nNOS, which forms NO and citrulline from arginine. NO diffuses freely across cell membranes where it interacts directly with intracellular signalling components. Acting presynaptically, NO can modulate the release of glutamate and GABA (Steinert et al., 2010). On the contrary, postsynaptic action of NO

increases the number of AMPA-Rs, which results in LTP (Malinow and Malenka, 2002). NO has been proposed as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanisms involved in LTP and LTD (Böhme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991)

. NO activates soluble guanylate cyclase (sGC) by binding to the heme site on the protein, inducing a conformational change (Bellamy et al., 2002). sGC in turn modulates the transformation of guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) (Garthwaite et al., 1988). cGMP acts as a second messenger and its production activates protein kinases (PKG), phosphodiesterases (PDE III) and cGMP-dependent kinase II (cGKII). An increase in AMPA-Rs has been reported to occur through cGKII binding to the CTD of GluR1. Blocking cGKII activity led to a reduction in LTP in hippocampal slices (Serulle et al., 2007). Besides, the NO/cGMP/PKG pathway has been shown to mediate actin depolymerisation and synaptic retraction through ROCK signalling in motor neurons (Sunico et al., 2010). Moreover, NO can regulate the Akt kinase signalling pathway and CREB (Calabrese et al., 2007). NO can also directly modify proteins through S-nitrosylation of MT associated protein (MAP) that has been shown to lead to growth cone collapse and axonal actin reconfiguration (Stroissnigg et al., 2007; Hardingham et al., 2013). For example, GluR1 AMPA-R subunit NO-mediated-S-nitrosylation has been linked to an increase in AMPA-R facilitating LTP and synaptic plasticity (Selvakumar et al., 2013).

BDNF has been shown to potently influence pre- and post-synaptic components of neural transmission in the cortex and hippocampus (Lu, 2003). BDNF enhanced the activation of post-synaptic NMDA-R but not AMPA-R and also increased the probability of pre-synaptic glutamate release in acute brain slices (Madara and Levine, 2008). There is a reciprocal and modulatory relationship between BDNF and NO influencing synaptic plasticity (Biojone et al., 2015). NO production can be stimulated by addition of BDNF (Koh et al., 1995; Samdani et al., 1997; Klöcker et al., 1998) and release of BDNF is inhibited by the NO/cyclic GMP signalling pathway in hippocampal slices (Canossa et al., 2002). Another study demonstrated that BDNF stimulated NO in the soma and dendrites via TrkB signaling and subsequent Ca^{2+} elevation in hippocampal neurons (Kolarow et al., 2014) involving an increase in nNOS activity (Biojone et al., 2015). However, endogenous BDNF wasn't able to increase intracellular NO levels and exert negative feedback on BDNF secretion (Kolarow et al., 2014) (**Fig 1.5**).

One possible mechanism of BDNF-induced NO increase could involve CREB-dependent genes, which then stimulate the dendritic arborisation and synaptic plasticity (Riccio et al., 2006; Nott et al., 2008; Hardingham et al., 2013). NO was able to negatively change BDNF affinity to the TrkB receptor and consequently decrease the impact of BDNF on the development of neuronal connections, as well as on synaptic strength as evidenced by LTP via the nitration or S-nitrosylation of amino acid residues in the amino acid sequence of BDNF (Biojone et al., 2015).

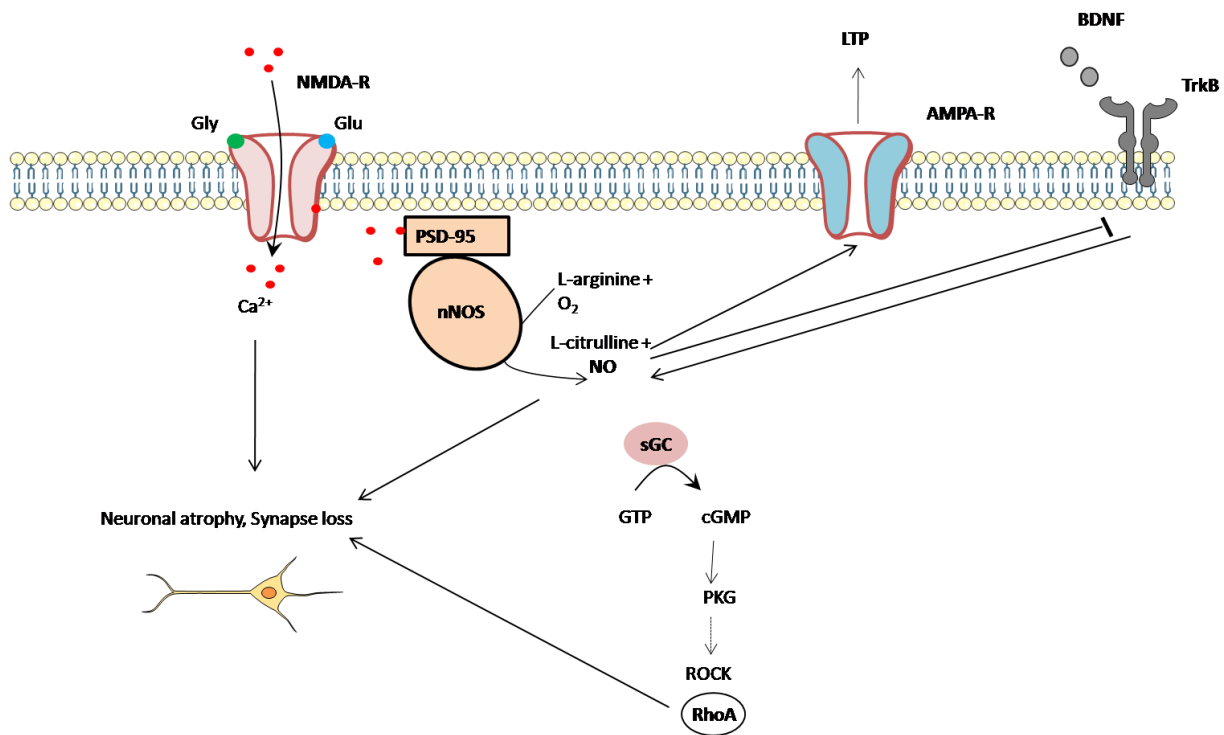


Figure 1.5: Glutamate as a regulator of outgrowth and synapse formation

Glutamate binds NMDA-R. (1) Ca²⁺ mediates cytoskeletal dynamics and filopodial extension, (2) PSD-95 and NO play roles in dendrite patterning and synaptic development, (3) NO mediates increase of AMPA-R and LTP induction, (4) NO/cGMP/PKG pathway via ROCK signalling mediates outgrowth and synapse formation, (5), NO downregulates BDNF whereas BDNF increases NO.

4. Neuronal atrophy in brain disorders

Neurotrophic factors have been found to play a major role in neuronal health and functional nervous system maintenance. Decreased neurotrophic signalling and subsequent neuronal impairment have been extensively studied in brain disorders. Research highlighted that nervous system disorders (developmental, neurodegenerative and psychiatric disorder) are accompanied by significant changes in dendritic branching or dendritic spine density [for review (Kulkarni and Firestein, 2012; Copf, 2016; Herms and Dorostkar, 2016)]. Such atrophy can lead to the loss of synaptic connections, and synaptic loss underlies many

neurological symptoms. Indeed, dysfunctional synapse development is highly implicated in neurodevelopmental disorders such as autism and Fragile X syndrome (McAllister, 2007).

4.1. Neurodevelopmental, genetic disorders

Neurodevelopmental and genetic disorders involve alterations in neuronal complexity and spine density (Kulkarni and Firestein, 2012; Copf, 2016). Autism is increasingly regarded as a disconnectivity syndrome with a decrease in dendritic branching in the hippocampus (Raymond et al., 1995) and an excess of spine number and synaptic connections in the cortex and hippocampus resulting from a deficit of synaptic pruning (Geschwind and Levitt, 2007; Bourgeron, 2009; Hutsler and Zhang, 2010; Bakos et al., 2015; Phillips and Pozzo-Miller, 2015). Magnetic resonance imaging (MRI) analysis in patients with autism also revealed that hippocampus and amygdala are enlarged in children with autism (Schumann et al., 2004). Similar changes were observed in other developmental, genetic disorders, such as Rett and Asperger, Fragile X and Down syndromes (Pardo and Eberhart, 2007). Rett syndrome presents a decrease on basal dendrites in the motor cortex and subiculum whereas both apical and basal dendrites are atrophied in the frontal cortex (Banerjee et al., 2012; Kulkarni and Firestein, 2012; Copf, 2016). Concerning Fragile X syndrome, there is an increase of long, thin and immature dendritic spines and synapses in the temporal cortex (Irwin et al., 2000; He and Portera-Cailliau, 2013). In contrast, Down syndrome shows a reduction in the number and length of dendritic branches with a reduction in spine and synapse density in the hippocampus (Fiala et al., 2002; Kurt et al., 2004).

In addition, evidence suggests that an excess of synaptic pruning in the pre-frontal cortex (PFC) contributes to the pathology of schizophrenia (Keshavan et al., 1994). Neurons present a reduced dendritic arbour, spine density and synaptic plasticity (Garey et al., 1998; Glantz and Lewis, 2000; Stephan et al., 2006, 2009; Bellon et al., 2011). Post-mortem analysis revealed a reduction in apical dendritic complexity in the subiculum (Rosoklija et al., 2000), impaired dendrite morphology in the PFC (Kalus et al., 2000), a decrease on basal dendrites in the medial PFC (mPFC) (Broadbelt et al., 2002) and altered dendritic arborisation in the CA3 (Cotter et al., 2000) with a decrease in dendritic spine volume (Kolomeets et al., 2005, 2007). In addition, several studies examining post-mortem brains have consistently reported a reduced volume of cerebral cortex (Suddath et al., 1989; Harvey et al., 1993; Zipursky et al., 1992, 1997).

4.2. Neurodegenerative disorders

In neurodegenerative disorders, synapse loss, rather than cell death, is increasingly regarded as the predominant neuronal pathology in Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Sunico et al., 2010). In Alzheimer's disease, there are reductions in the apical and basal dendritic lengths in the CA1 and a decrease in the dendrite length of apical dendrites in the DG. Neuronal loss and synaptic impairment also play a role in the cognitive decline associated with Alzheimer's disease (He and Shen, 2009; Palop and Mucke, 2010). Similar impairments are observed in Parkinson's disease with neuronal loss and decreased spine/synapse density in the substantia nigra (Day et al., 2006; Fiala et al., 2002). Neuronal impairments are also prominent in Huntington's disease. Analyses of striatal spiny neurons patients at mild stages of the disease revealed an increase in dendritic branching and spine density whereas at a later stage, patients presented a truncation of dendritic arbours, and decreases in spine density (Graveland et al., 1985; Ferrante et al., 1991).

4.3. Neuropsychiatric disorders

In neuropsychiatric disorders, pre-clinical and clinical research in anxiety and depression disorders suggest that these stress-related disorders are associated with neuronal atrophy, reduced number of spines and dendritic arbours in some brain regions (Popoli et al., 2012; McEwen and Morrison, 2013; Duman, 2014; Ménard et al., 2016)

In patients, depression leads to morphometric changes, including a reduction of the hippocampus and PFC volume and a loss of neurons (Rajkowska et al., 1999; Bianchi et al., 2005; Warner-Schmidt and Duman, 2006). Other studies demonstrate a fall in the number of spine synapses in the PFC (Kang et al., 2012) and a decrease of synapse-related genes in the hippocampus of depressed subjects (Duric et al., 2013). In addition, depressed patients present reduced dendrite and spine density in the CA3 region of the hippocampus (Soetanto et al., 2010). The characterisation of the cellular determinants underlying these changes in patients is limited. However, animal models have provided evidence that chronic stress induces alterations in the complexity and function of spines and synapses in brain areas implicated in anxiety and depression (Ronald S Duman et al., 2016).

5. Major depressive disorder

5.1. Depression

Major depressive disorder (MDD) is a common psychiatric disorder that affects 350 million people of all ages and more women than men (WHO, 2015). According to WHO, depression is now the current the leading cause of disability worldwide. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), a MDD is diagnosed through at least five of the following symptoms, persisting for at least two weeks: depressed mood, loss of interest or pleasure, significant weight loss, changes in appetite, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue or loss of energy, feelings of worthlessness or excessive or inappropriate guilt, diminished ability to think or concentrate, indecisiveness, recurrent death and suicidal thoughts (American Psychiatric Association, 2013).

5.2. Volumetric and structural brain alterations in depression

Meta-analyses of structural MRI studies indicate volumetric changes in brain areas of MDD patients relative to healthy controls. MDD patients have a smaller hippocampus (Sheline et al., 1996; Campbell and MacQueen, 2004; Videbech and Ravnkilde, 2004; Kempton et al., 2011; Arnone et al., 2012; Schmaal et al., 2016), which can be observed after the first episode of depression (Cole et al., 2011), a reduction in the volume of the PFC, dorsomedial PFC (dmPFC), orbitofrontal, cingulate cortices and striatum (Arnone et al., 2012; Bora et al., 2012; Sacher et al., 2012; Lai, 2013). Other studies showed that the amygdala volume is reduced in MDD (Mervaala et al., 2000; Hamilton et al., 2008). In addition, changes in the hippocampal volume are sensitive to antidepressant treatment, with an increase in volume following traditional antidepressant (Frodl et al., 2008) and electroconvulsive therapies (ECT) (Abbott et al., 2014). There is a HPA axis overactivity in a subset of depressed patients (Varghese and Brown, 2001). Smaller hippocampus in MDD has been associated with an increase in glucocorticoid receptor activation in the peripheral blood, suggesting that structural changes can be associated with functional alterations in the hypothalamic pituitary adrenal (HPA) axis function (Frodl et al., 2012).

Diffusion tensor imaging (DTI) allows the assessment *in vivo* of white matter microstructural integrity through the measurement of the restriction in the diffusion of water molecules in brain tissue (Moser et al., 2009). Several DTI studies in patients with MDD have reported reduced cortical fractional anisotropy (FA), a measure of white matter integrity and fiber directionality in the frontal and temporal lobes [for review, (Sexton et al., 2009)] as well as in

the amygdala (Arnold et al., 2012) suggesting that MDD may be a disconnection syndrome. Indeed, there is an impairment in the white matter fascicles connecting the PFC with other cortical areas (frontal, temporal and occipital lobes), subcortical areas (amygdala and hippocampus) (Liao et al., 2013), thalamus and striatum (Drevets, 2000).

Although this thesis is mainly focused on the PFC, comparatively fewer studies have been done in this region compared to the hippocampus. There is a loss of PFC gray matter associated with chronic stress. Structural imaging has shown that the number of adverse events a person has been exposed to correlates with smaller PFC gray matter (Ansell et al., 2012). Chronic stress in humans also weakens PFC functional connectivity (Liston et al., 2009), and PFC regulation of the amygdala (Kim et al., 2013). Thus, sustained stress exposure leads to more persistent changes in brain circuits regulating behavior and emotion. Interestingly, the reduced volume of certain brain regions in depression is associated with a decrease in gray matter [for reviews (Stockmeier and Rajkowska, 2004; Czéh and Lucassen, 2007; Duman, 2009)]. One report suggests that the size of neurons in the hippocampus is reduced (Stockmeier et al., 2004a). As neuropil comprises dendrites, axons as well as glial processes, a loss of neuropil could reflect a reduced dendritic branching, reduced spine density and complexity with smaller glial processes and contribute to the decrease in hippocampal volume observed in MDD. The hypothesis of neuropil reduction in the hippocampus in MDD is supported by other post-mortem studies revealing a decrease in dendritic spine density on neurons and diminished arborisation of apical dendrites in the subiculum in a small group of mixed subjects with bipolar disorder or depression (Rosoklija et al., 2000).

5.3. Functional brain alterations in depression

Depression has been associated with changes in neurotransmitter systems (Crow et al., 1984; Cheetham et al., 1989; Yildiz-Yesiloglu and Ankerst, 2006; Rajkowska and Stockmeier, 2013). Magnetic resonance spectroscopy (MRS) in MDD patients have shown a reduction of glutamate (Yildiz-Yesiloglu and Ankerst, 2006; Luykx et al., 2012; Miladinovic et al., 2015), a decrease of glutamine (Martinez-Hernandez et al., 1977) and GABA in the PFC compared to healthy controls (Walter et al., 2009; Pehrson and Sanchez, 2015). These transmitters are increased in the amygdala, orbito frontal cortex, medial thalamus (Drevets, 2000) and anterior cingulate cortex (Sacher et al., 2012), which are involved in emotional behaviour and

stress response. In addition, positron emission tomography (PET) demonstrated that the regional cerebral blood flow and glucose metabolism are impaired in MDD.

Functional MRI (fMRI) has been used to characterise the normal and pathological brain activation based on the Blood Oxygen Level Dependent (BOLD) signal. Although the BOLD signal is described as an indirect measure of neuronal activity, it matches neuronal activation (Siero et al., 2014). Resting state fMRI in the absence of task execution revealed that MDD patients present a lower dorsolateral PFC activation (Alcaro et al., 2010), and an increased baseline activity in the orbitofrontal and subgenual anterior cingulate cortices [for review, (Hasler and Northoff, 2011)] relative to controls. MDD patients also showed hyperactivity in the default mode network (set of brain regions which are more active at rest than during task execution) including the orbital frontal cortex, the mPFC/anterior cingulate cortex, the lateral temporal cortex, the inferior parietal lobe, the posterior cingulate and retrosplenial cortex, the hippocampus and parahippocampal cortex (Lu et al., 2012) [for review, (Whitfield-Gabrieli and Ford, 2012)]. In addition, fMRI revealed an abnormal activation of the PFC during emotional experience (Steele et al., 2007) and performance in a cognitive task in MDD (Zhang et al., 2013).

5.4. Animal models of depression

Animal models of depression have been developed to screen for novel antidepressant drugs and understand the neurochemical and physiological impairments which may underlie depressive behaviours. Developing an animal model of depression is a real challenge. Validity criteria for animal models of depression can be determined by the etiological relevance of how the model was constructed relative to the human disease (construct validity), the accuracy with which the model reproduces anatomical, biological and behavioural markers which are evident in the human disease (face validity), and the capacity of currently used treatment for depression to reverse behavioural alterations (predictive validity) (McKinney and Bunney, 1969; Willner, 1984; Belzung and Lemoine, 2011).

Among the animal models of depression, some models are based on stress as stress is a risk factor for depression. Those models include the early life stress model [for review, (Cryan and Holmes, 2005)] or chronic unpredictable stress (CUS) are well established [for review, ((Willner, 1997)]. Early life stress models involve stress during pregnancy or maternal separation during the juvenile period. It is mainly associated with a high level of corticosterone and HPA axis activation. The behavioural impairments created by early life

stress can be reversed by current antidepressant treatments (Murgatroyd, 1970; Louvart et al., 2009). Chronic mild stress (CMS) provokes anhedonia, sexual impairment and depressive-like behaviour in a range of several tests which can be reversed by antidepressant treatment (Papp et al., 1996; Harkin et al., 2002a; Nestler et al., 2002b). However, it is not possible to accurately model all of the symptoms of depression in a rodent model.

Chronic stress paradigms, inbred animals with high levels of anxiety/depressive-like behaviours have been shown to produce changes in rodent brain morphology [for review, (McIntosh et al., 2017)]. The PFC is one of the brain regions that is most sensitive to the detrimental effects of stress, it provides top-down regulation of behavior, thought and emotion, generating the mental representations needed for flexible, goal-directed behavior, including the ability to inhibit inappropriate impulses, regulation of attention, reality testing, and insight about one's own and others' actions (Arnsten, 2009). Chronic stress exposure leads to dendritic atrophy in PFC (Arnsten et al., 2015). In addition, increased lateral ventricular volume and decreased PFC and hippocampal volume appear to be relatively consistent hallmarks in both MDD patients and animal models of depression, adding to their face validity.

5.5. Effect of stress on neuronal plasticity

5.5.1. Effect of stress on neuronal complexity

There is emerging evidence linking stress-related changes in dendritic spine density homeostasis, dendrite arborisation and neuronal complexity with the pathophysiology of stress-related psychiatric disorders such as depression (Duman and Duman, 2015; Sato, 2013). Exposure to stressful life events is a major risk factor associated with the onset of depression (Kessler, 1997). Both animal and human studies revealed that stress and depression are associated with neuronal atrophy and dendritic spine loss in the PFC and hippocampus [(Popoli et al., 2012; McEwen and Morrison, 2013; Duman and Duman, 2015), **for details refer to table 1.1**]. Specifically, chronic stress reduces the neuronal complexity (length and number of intersections) in both apical and basal dendrites of interneurons, granule or pyramidal cells in the hippocampus in mice, rats and adult male tree shrews (CA1, CA3 and DG) (Watanabe et al., 1992; Magariños et al., 1996; Sousa et al., 2000; Bessa et al., 2008; 2013; Chen et al., 2016; Gilabert-Juan et al., 2017). Similar effects have been observed after acute or chronic stress in the PFC with a preferential decrease in the length of apical dendrites in rats (Cook and Wellman, 2004; Liston et al., 2006; Radley et al., 2006; Radley et

al., 2013; Moench et al., 2016). Interestingly, chronic stress causes an opposite effect in the amygdala with an increase in neuronal complexity (length and intersections) in rats and mice (Vyas et al., 2002;2004; Padival et al., 2013, 2015; Lau et al., 2017) but also in the nucleus accumbens in rats (Taylor et al., 2014). Equivocal results have been reported concerning the striatum with either an increase or decrease in neuronal complexity following chronic stress in rats (Dias-Ferreira et al., 2009; Taylor et al., 2014).

During stress, the HPA axis is activated and causes the release of a number of neuroactive substances such as glucocorticoids. Hypercortisolemia in depressed patients has been reported in a number of studies (Tata et al., 2006). Chronically elevated levels of corticosterone, the primary glucocorticoid in mice, have been shown to reduce neuronal outgrowth and promote synaptic loss in the CA3 (Tata et al., 2006). In the hippocampus, chronic corticosterone administration produced a similar effect to chronic stress with a decrease in intersections and dendritic length in mature granule cells in mice and rats (Gourley et al., 2013; Yau et al., 2016), but also with an atrophy of both apical and basal dendrites evident in the PFC in rats (Cerqueira et al., 2007).

Table 1.1: Effect of stress on neuronal complexity in animal models of depression

Structure	Substructure	Model of stress	Neuronal complexity	Reference
Hippocampus	Dentate gyrus (Adult granule cells)	Chronic social defeat stress, adult C57BL/6 mice	Decrease length and branching	(Chen et al., 2016)
	Dentate gyrus and CA1 (Granule and pyramidal cells)	Chronic unpredictable stress, male Wistar rats	Dendritic atrophy	(Bessa et al., 2008) (Sousa et al., 2000)
	Dentate gyrus (Granule cells)	Chronic corticosterone administration, adult male Sprague-Dawley rats	Decreased intersections and dendritic length in mature, but not young, granule cells	(Yau et al., 2016)
	CA1 (Pyramidal cells)	Chronic corticosterone exposure, Male C57BL/6 mice	Basal dendritic atrophy	(Gourley et al., 2013)
	CA1 and CA3 (Interneurons)	Repeated restraint stress, adult male GIN mice (EGFP-expressing inhibitory neurons, Tg(GadGFP-45704Sw n))	Decrease in number of intersection in the CA1 but not in the CA3	(Gilabert-Juan et al., 2017)
	CA3 (Pyramidal cells)	Repeated restraint stress and psychological stressors, adult male tree shrews (<i>Tupaia belangeri</i>), rats	Decreased length and branching of apical dendrites	(Magariños et al., 1996) (Watanabe et al., 1992)
Prefrontal cortex	Layers II/III and V in mPFC (Pyramidal cells)	Repeated restraint stress, male rats, adult male Sprague Dawley albino rats	Atrophy of apical dendrites (more sensitive) but also basal dendrites	(Cook et al. 2004) (Radley et al. 2006)
		Chronic corticosterone exposure, adult male Wistar rats, adult male Sprague-Dawley rats	Atrophy of apical dendrites (more sensitive) but also basal dendrites	(Cerqueira et al., 2007) (Wellman, 2001)
	Layers II/III in PFC (Pyramidal neurons)	Mild traumatic brain injury, C57Bl/6J male mice	No effect on neuronal complexity (number of intersections)	(Winston et al., 2016)
	Lateral orbitofrontal cortex	Repeated restraint stress, male Sprague Dawley rats	Apical dendritic hypertrophy	(Liston et al., 2006)
	Pre-limbic subdivision (Pyramidal neurons)	Repeated variable stress, adult male Sprague Dawley albino rats	Decrease in apical length and branching	(Jason J Radley et al., 2013)
	Infralimbic cortex (Pyramidal neurons)	Acute stress, adult male Sprague Dawley rats	Decrease apical dendritic length	(Moench et al., 2016)

Other structures	Basolateral amygdala	Chronic immobilisation stress Chronic unpredictable stress, male Wistar rats	Increased dendritic arborisation	(Vyas et al., 2004, 2002)
		Repeated restraint stress, male Sprague-Dawley rats	Increase of dendritic length in the basal nucleus but no effect in the Lateral nucleus in adult rat.	(Padival et al., 2013, 2015)
		Repeated restraint stress, C57BL/6N male mice	Decrease in length and number of intersections of MeA stellate neurons and increase in length and intersection of pyramidal neurons.	(Lau et al., 2017)
	Bed nucleus of the stria terminalis	Chronic immobilisation stress Chronic unpredictable stress, male Wistar rats	Dendritic hypertrophy	(Feldman et al., 1990) (Vyas et al., 2002)
	Dorsal medial striatum and dorsolateral and dorsomedial striatum (Medium spiny neurons)	Chronic unpredictable stress, rats	Dendritic atrophy	(Dias-Ferreira et al., 2009)
		Chronic variable stress, male Sprague-Dawley rats	Increased dendritic complexity in the dorsolateral striatum but no effects in dorsomedial striatum.	(Taylor et al., 2014)
	Nucleus accumbens	Chronic variable stress, male Sprague-Dawley rats	Increased dendritic complexity in nucleus accumbens core and decreased complexity in shell	(Taylor et al., 2014)

5.5.2. Effect of stress on dendritic spines

At a different level, stress also affects dendritic spine density (Duman and Duman, 2015), **for details refer to table 1.2**]. Spines are remarkably dynamic, changing size and shape over time scales ranging from seconds to days (De Roo et al., 2008). Each spine has a PSD at its tip that becomes aligned with the pre-synaptic membrane of a target axon terminal. The formation of spines may help to compartmentalise excitatory intracellular activity and the differing morphology may play a role in the regulation of the communication between synapses and dendrites (Hering and Sheng, 2001). Spines can be altered following exposure to stress, supporting the hypothesis that changes in neuronal and dendritic complexity are involved in the pathogenesis of CNS disorders (Hering and Sheng, 2001; Sato, 2013; Duman and Duman, 2015). Stress-induced changes in the density of spines and synapses have been reported in a number of regions including the PFC and the hippocampus implicated in the pathophysiology of depression.

There is a reduction in the number of spines in the CA1 and CA3 regions of the hippocampus following chronic stress in rats and mice (Magariños et al., 1997; Sousa et al., 2000; Pawlak et al., 2005; Kassem et al., 2013; Qiao et al., 2014; Morales Rico et al., 2015; García-Rojo et al., 2017) or pre-natal stress in rats (Muhammad et al., 2012; Murmu et al., 2006). However, two other studies contested these findings with no effect or an increase in spines following chronic stress in mice and adult male tree shrews (Gilabert-Juan et al., 2017; Magariños et al., 1996; Sunanda et al., 1995). Interestingly, chronic corticosterone administration reproduced the effect of chronic stress on spines in the CA1 in mice (Gourley et al., 2013) but not in the DG in rats (Yau et al., 2016). Chronic stress appears to affect stubby spines and filopodia in the hippocampus in rats (García-Rojo et al., 2017) and chronic corticosterone administration affects thin and stubby spines in mice (Gourley et al., 2013; Wang et al. 2013).

In the PFC, chronic stress reduces spine density in rats (Michelsen et al., 2007; Liu and Aghajanian, 2008; Radley et al., 2008; Bloss et al., 2011; Li et al., 2011; Radley et al., 2013; Luczynski et al., 2015; Anderson et al., 2016). Lower spine density was also found with maternal separation or pre-natal restraint stress in rats (Murmu et al., 2006; Muhammad and Kolb, 2011; Mychasiuk et al., 2012), social defeat in mice (Shu and Xu, 2017), social isolation in mice (Castillo-Gómez et al., 2017) and single traumatic brain injury (TBI) in mice (Winston et al., 2016). Interestingly, the type of stress can influence the effect on spines. Chronic restraint stress (CRS) was shown to preferentially induce a loss of small spines (thin and stubby) in the PFC in rats (Bloss et al., 2011; Jason J Radley et al., 2008), although one study found a decrease in both small and large spines in rats (Luczynski et al., 2015), whereas CUS produced a loss of large spines in this area in rats (Michelsen et al., 2007; Li et al., 2011; Radley et al., 2013). Chronic corticosterone treatment impaired small spines in rats (Anderson et al., 2016). Overall, these studies indicate a shift from large to small synapses or attenuation of synapse development in response to stress (Duman and Duman, 2015).

In contrast, dendritic hypertrophy was found in the amygdala in response to CRS in rats and mice (Vyas et al., 2002; Padival et al., 2013; Yi et al., 2017) and social defeat in mice (Christoffel et al., 2012). No effect was found in response to social isolation in mice (Castillo-Gómez et al., 2017). Chronic corticosterone treatment increased spine and synapse density on pre-limbic cells that project onto the bed nuclei of the stria terminalis (BNST) in response to elevated corticosterone in mice (Gourley et al., 2013). In the nucleus accumbens, chronic stress caused an increase in spine density on medium spiny neurons following pre-natal stress or maternal separation in rats (Muhammad et al., 2012), and emotional (exposure

to a CD1 mouse in an empty compartment) or physical stress (exposure to a CD1 mouse in the same compartment) in mice (Warren et al., 2014). To conclude, stress seems to reduce neuronal complexity and spines in the pre-frontal area and hippocampus, but increases them in the amygdala and nucleus accumbens. Interestingly, apical dendritic neuronal segments also seem to be more affected by stress than basal segments in the brain (Duman and Duman, 2015).

Table 1.2: Effect of stress on dendritic spine density in animal models of depression

Structure	Substructure	Model of stress	Spines	Reference
Hippocampus	Dentate gyrus (Granular cells)	Chronic corticosterone administration, adult male Sprague-Dawley rats	No effect on spines density	(Yau et al., 2016)
	CA1 (Granular cells and pyramidal neurons)	Chronic corticosterone administration, Male C57BL/6 mice, WT mice	Spine density loss (thin and stubby)	(Gourley et al., 2013) (Wang et al., 2013)
		Chronic restraint stress, male Sprague-Dawley rats	Decrease in stubby spines and filopodia spine density in pyramidal neurons	(García-Rojo et al., 2017)
	Ventral CA1	Chronic restraint stress, female Long Evans rats	Decrease in basal spine density in females but not in males	(Morales Rico et al., 2015)
	CA1 and CA3	Chronic restraint stress, mice	Spine density loss	(Kassem et al., 2013) (Qiao et al., 2014)
		Chronic unpredictable mild stress, male rat		
	C3g (Pyramidal neurons)	Chronic restraint stress, GIN mice	No effect on spine density	(Gilabert-Juan et al., 2017)
		Prenatal (PS) and maternal separation (MS), rats	PS decrease spine density and MS increased apical spine density	(Muhammad et al., 2012)
	CA3 (Pyramidal cells)	Repeated psychosocial or restraint stress	Spine density loss	(Magariños et al., 1997) (Sousa et al., 2000)
		Chronic unpredictable normal and mild stress		(Pawlak et al., 2005)
		Adult male Sprague-Dawley rats male, C57/BL6 mice, Wistar rats, male rats		(Qiao et al., 2014)
		Repeated psychosocial or restraint stress, adult male tree shrews (<i>Tupaia belangeri</i>)	Increase in spine density	(Magariños et al., 1996)

Prefrontal cortex		Chronic restraint stress, rats	No effect in spine density	(Sunanda et al., 1995)
	Layer II/III PFC (Pyramidal neurons)	Repeated restraint stress, adult male Sprague-Dawley albino rats	Decrease in spine volume and surface area	(Radley et al., 2008)
	Layers II/III and V of the PFC (Pyramidal neurons)	Maternal separation Prenatal chronic restraint stress, rats, long evans rats	Decrease in basal dendritic spine density	(Muhammad and Kolb, 2011) (Murmu et al., 2006) (Mychasiuk et al., 2012)
		Single traumatic brain injury, C57Bl/6J male mice	Time dependent effect on spine density, decrease after 12 or 24 h with an increase at 72 h.	(Winston et al., 2016)
		Repeated mild restraint stress, adult male albino rats	Reduction in spine density on apical dendrites and most prominent distally	(Liu and Aghajanian, 2008)
		Chronic unpredictable stress, male Sprague-Dawley rats	Decrease in large-diameter mushroom spines	(Li et al., 2011)
		Social defeat, Thy1-YFP-H line on C57Bl6 background	Decrease in spine formation after the defeat with an increase in the maintenance of newly formed spines 10 days post defeat.	(Shu and Xu, 2017)
		Maternal separation, Male White-Wistar rat pups, rats	Decrease in basal dendritic spine density	(Bock et al., 2004) (Gos et al., 2008)
	Medial PFC	Prenatal stress, Sprague-Dawley rats	Large spine density reduction on basal dendrites	(Michelsen et al., 2007)
	PFC	Repeated restraint stress, adult male Sprague-Dawley albino rats	Reduction in small spine density on basal dendrites	(Radley et al., 2008)
		Chronic corticosterone treatment, male Sprague Dawley albino rats	Reduction in thin spine density	(Anderson, 2016)
		Chronic restraint stress, male Sprague Dawley rats	Decrease in thin and stubby spine density	(Bloss et al., 2011)
		Social isolation (from age of weaning), GIN mice, Thy1 mice	No effect on spine density on pyramidal neurons but increase of spine density in interneurons	(Castillo-Gómez et al., 2017)
		Chronic restraint stress, rats	Decreased in thin and mushroom spine density	(Luczynski et al., 2015)

			preferably on left PFC callosal neurons	
		Chronic unpredictable stress, adult male Sprague Dawley albino rats	Decrease in mushroom spine density	(Radley et al., 2013)
Other structures	Basolateral amygdala	Chronic immobilisation stress, male Wistar rats	Increase in spine density	(Vyas et al., 2002)
	Basolateral amygdala	Repeated restraint stress, male Sprague-Dawley rats	Focal increase of spines in LAT neurons of adult rats whereas, decrease of spines in adolescent rats. In BA neurons of adult rats, spines increase and spines decrease in adolescent.	(Padival et al., 2015)
	Nucleus Accumbens	Social isolation (from age of weaning), GIN mice, Thy1 mice	No effect on spine density on Interneurons	(Castillo-Gómez et al., 2017)
		Social defeat stress, C57BL/6J mice	Increase in spine density	(Christoffel et al., 2012)
		Chronic restraint stress, mice	Increase in spine density	(Yi et al., 2017)
	Prelimbic cells that project to the bed nuclei of the stria terminalis	Chronic corticosterone treatment, Male C57BL/6 mice	Increase in spine density	(Gourley et al., 2013)
	Nucleus accumbens	Prenatal (PS) and maternal separation (MS), rats	Increase in spine density in medium spiny neurons	(Muhammad et al., 2012)
		Subchronic variable stress, female mice	No effect on spine density	(Brancato et al., 2017)
		Emotional and physical stressor, c57BL/6J mice	Increase in spine density during adolescence for both stressors but only physical stress increase spine density in adulthood	(Warren et al., 2014)

6. New directions for the treatment of depression

6.1. Current pharmacological treatments

The first antidepressant treatments were the mono amine oxidase (MAO) inhibitors (e.g., Tranylcypromine, Phenelzine). Their mechanism of action is based on a reduction of the enzymatic breakdown of serotonin and noradrenaline (Schildkraut, 1965; Berton and Nestler, 2006). Selective serotonin reuptake inhibitors (SSRIs) (e.g., fluoxetine, citalopram and vortioxetine) are the most commonly used drugs to treat depression. SSRIs are believed to increase the extracellular level of the neurotransmitter serotonin by limiting its

reabsorption (reuptake) into the presynaptic cell, increasing the level of serotonin in the synaptic cleft available to bind to the postsynaptic receptor.

Tricyclic antidepressants (e.g., imipramine and desipramine) are norepinephrine-serotonin reuptake inhibitors, which inhibit the plasma membrane transporters for serotonin and/or noradrenaline. Newer classes of antidepressants include serotonin and noradrenaline reuptake inhibitors (SNRIs) (e.g., venlafaxine and duloxetine), selective serotonin reuptake inhibitors (SSRIs) (e.g., fluoxetine and citalopram), acting as serotonin (5-HT) agonists, and selective noradrenaline reuptake inhibitors (NRIs) (e.g., atomoxetine and reboxetine).

SSRIs, NRIs, SNRIs are ineffective on patients who are resistant to tricyclic antidepressants because they share a common mechanism of action (**Table 1.3**). For this reason, there is a need to develop new treatments for depression for treatment-resistant patients. Recent studies have focused on the effect of drugs targeting the glutamatergic pathway and the use of NMDA-R antagonists to treat depression. A single intravenous injection of ketamine, a non-competitive NMDA-R antagonist, has been shown to reduce depressive symptoms within hours (Berman et al., 2000). However, there are side effects. Finding a more specific drug with fewer side effects and targeting treatment-resistant patients remains a challenge.

Table 1.3. Mechanisms of action of antidepressants

Drug class	Drugs	Mechanism of action
Monoamine oxydase inhibitors (MAO)	- Iproniazid - Phenelzine - Tranylcypromine - Isocarboxazid - Selegiline - Marsilid - Nardil - Parnate - Marplan - Emsam, Eldepryl - Moclobemide - Manerix, Moclamine	Irreversible and reversible of MAO-A and MAO-B that increases noradrenergic and serotonergic neurotransmission by inhibiting the catabolism of norepinephrine and serotonin.
Serotonin, norepinephrine reuptake inhibitors (SNRIs)	- Amitriptyline - Venlafaxine - Doxepin - Duloxetine	Increases noradrenergic and serotonergic neurotransmission by blocking the norepinephrine and serotonin transporters at the presynaptic terminal
Secondary amine tricyclics, Tricyclic antidepressants (TCAs)	- Imipramine - Dexipramine - Clomipramine - Trimipramine	
Selective serotonin reuptake inhibitors (SSRIs)	- Fluoxetine - Fluvoxamine - Paroxetine - Sertraline - Citalopram - Escitalopram - vortioxetine	Blockade of serotonin reuptake results in enhanced and prolonged serotonergic neurotransmission
Noradrenaline re-uptake inhibitors (NRIs)	- Atomoxetine - Reboxetine - Edronax	Inhibits reuptake of norepinephrine

6.2. Non pharmacological treatments of depression

There are alternative therapies for treatment-resistant patients (Holtzheimer and Mayberg, 2011). One of the major and most promising treatments, ECT is based on the delivery of an electric current that induces a seizure. Although there is a remission rate greater than 50%, ECT is associated with amnesia during the weeks following treatment (Akhtar et al., 2016). Another treatment, deep brain stimulation, is an invasive technique that promotes an antidepressant effect by stimulating brain areas implicated in depression (Holtzheimer and Mayberg, 2011; Schlaepfer et al., 2013). Areas targeted are the subcallosum cingulate (brodmann's area 25), ventral capsule, ventral striatum, NAC and medial forebrain bundle. In addition, in rats, high-frequency stimulation of the ventromedial prefrontal cortex has been associated with antidepressant-like effects using the FST (Hamani et al., 2010).

Based on a similar mechanism but non-invasive, the trans-cranial magnetic stimulation targets small regions of the brain using magnetic fields to stimulate nerve cells in the brain to reduce symptoms of depression. It targets one area of the brain that has been particularly weakened in people with depression, the dorsal lateral prefrontal cortex (DLPFC). Similarly, vagal nerve stimulation delivers electrical impulses to the vagus nerve. A category of

treatment based on psychological support has emerged. For example, cognitive behavioural therapy is commonly used and the therapist helps the patient to understand perceptions of self and how behaviour can influence thoughts and emotions. Psychological support is generally combined with pharmacological treatment (Eisendrath et al., 2016).

6.3. Neurobiology of depression

6.3.1. Mono-amine hypothesis

The first theory of depression postulates that symptoms of MDD originate from underactivity of monoamines. It was formulated in the 1960s, when deficiencies in serotonin, noradrenaline levels but also the dopamine system were found to be linked to depression (Czéh and Di Benedetto, 2013). However, antidepressant drugs targeting monoamines are not effective in all patients (50 to 60% don't respond to the first anti-depressant treatment) and some of them are left with residual symptoms and psychosocial disability (Trivedi et al., 2006).

6.3.2. Glutamatergic hypothesis

As the drugs targeting the monoamine pathway are not always effective, developing new drugs to treat depression is necessary. Glutamate-based research has provided novel insights into the pathogenesis of MDD (Mathews et al., 2012). As glutamatergic synapses represent 85% of cortical synapses, they are involved in several brain functions [for review, (Sanacora et al., 2012)]. Glutamatergic transmission is known to be responsible for many key functions of the brain (Pessoa, 2008) including cognition (Niciu et al., 2012; Citri and Malenka, 2008). Depressed patients have higher glutamate and glutamine levels in the plasma compared to healthy subjects (Küçükibrahimoğlu et al., 2009). Interestingly, antidepressant drugs have been shown to decrease plasma glutamate levels in depressed individuals (Mitani et al., 2006; Küçükibrahimoğlu et al., 2009; Niciu et al., 2012). However, BBB has a very low permeability to glutamate. The BBB essentially prevents the entry of glutamate into the CNS, and most of the glutamate that is present in the brain is synthesised by astrocytes (Nedergaard et al., 2003). It is thus difficult to link plasma and brain glutamate concentrations.

MRS studies have found an impairment of glutamate and glutamine levels in different brain regions (Yüksel and Öngür, 2010) with an increase of the glutamate-glutamine ratio in the occipital cortex (Sanacora et al., 2004) and a decrease in the PFC (Hasler et al., 2007; Michael et al., 2003b), anterior cingulate cortex (Auer et al., 2000; Zhang et al., 2013) and left amygdala (Michael et al., 2003a).

Glutamatergic dysfunction such as hyperactivity can lead to neurotoxicity and cell death when neuronal glutamate receptors are activated for an extended period. As glial cells play a major role in glutamatergic level modulation by removing excess of glutamate from the synapse, a glutamate level impairment may be astrocytic in origin (Niciu et al., 2012).

6.4. Targeting the glutamatergic system to develop novel therapeutics

6.4.1. Glutamatergic receptors

Glutamate is the most abundant neurotransmitter in the mammalian brain and plays an important role in excitatory neurotransmission. It involves several receptors divided into two categories: ionotropic and metabotropic receptors (Ghasemi et al., 2014) (**Fig 1.6**), (**Table 1.4**).

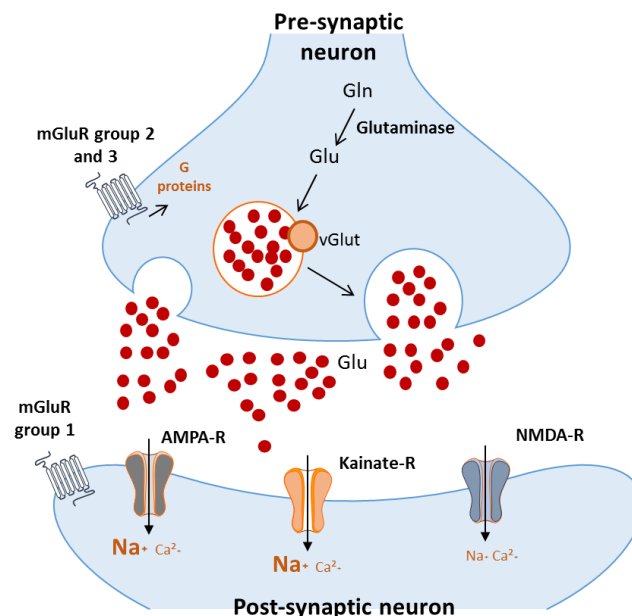


Figure 1.6: Glutamatergic synapse

Ionotropic receptors are located on post-synaptic neurons while metabotropic receptors are located pre and post-synaptically (mGluR group 1 is post-synaptic and group 2 and 3 are pre-synaptic). Ionotropic receptors are ligand-gated receptors that allow cation [calcium (Ca^{2+}) and/or sodium (Na^{+})] influx upon glutamate binding. Metabotropic receptors are G-protein-coupled receptors.

Table 1.4: Glutamate receptors properties

Glutamate involves several receptors divided into 2 categories: ionotropic and metabotropic receptors. Metabotropic glutamate receptors (mGluR1-8) are G-protein-coupled receptors and play a role in cell excitability modulation and synaptic transmission via second messenger signalling pathways (G protein: excitatory Gq or inhibitory Gi/G0) leading to the activation of the phospholipase C/inositol 1,4,5-triphosphate/diacylglycerol (PLC/IP3/DAG) pathway or inhibition of adenylate cyclase (AC). Ionotropic glutamate receptors (iGLURs) form an ion channel pore. They include AMPA, kainate and NMDA receptors. They are ligand-gated receptors that allow cation [calcium (Ca²⁺) and/or sodium (Na⁺)] influx upon glutamate binding.

Receptor	Protein Subunit	Location	Receptor Properties
Ionotropic Receptors			
NMDA-R	NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B	Post-synaptic	• Heterotetrameric; • High Ca²⁺ permeability, • Low Na⁺ permeability • Long channel open time
AMPA-R	GluR ₁ , GluR ₂ , GluR ₃ , and GluR ₄	Post-synaptic	• Heterotetrameric • High Na⁺ permeability, • Low/moderate Ca²⁺ permeability, • Short channel open time
Kainate receptor	GluR ₅ , GluR ₆ , GluR ₇ , and KA2	Post-synaptic	• Homotetrameric or heterotetrameric; • Low Ca²⁺ permeability • short channel open time
Metabotropic Receptors			
Group 1	mGluR ₁ and mGluR ₅	Post-synaptic	• Homodimeric; • Gq activates PLC, IP3, DAG
Group 2	mGluR ₂ and mGluR ₃	Pre-synaptic	• Homodimeric; • Gi/o inhibit adenyl cyclase and cAMP
Group 3	mGluR ₄ , mGluR ₆ , mGluR ₇ , and mGluR ₈	Pre-synaptic	• Homodimeric; • Gi/o inhibit adenyl cyclase and cAMP

6.4.1.1. Metabotropic glutamate receptors

mGluR1-8 are membrane-bound proteins that are activated by extracellular ligands such as light, peptides and neurotransmitters, and transduce intracellular signals via interactions with G proteins (Niswender and Conn, 2010). They modulate cell excitability and synaptic transmission via second messenger signalling pathways (Gprotein: Gq, Gi, Go) throughout the central nervous system (CNS). Group I includes mGluRs 1 and 5, they are homodimeric and located in the post-synaptic neuron. Group II includes the homodimeric mGluRs 2, and group III the homodimeric mGluRs, 4, 6, 7 and 8; they are both located in the pre-synaptic neuron. Groups II and III are negatively modulating excitatory neurotransmission upon activation whereas group I modulates activation.

6.4.1.2. Ionotropic receptors

iGluRs are widely distributed through the CNS in post-synaptic neurons and form an ion channel pore. They include AMPA, kainate and NMDA receptors. These glutamate receptors are named after the agonists that activate them: NMDA (*N*-methyl-D-aspartate), AMPA (α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and kainate receptors (kainic acid). They are non selective cation channels, allowing the passage of Na^+ and K^+ and in some cases small amounts of Ca^{2+} . They contain an extracellular N terminal domain, three transmembrane domains (M1, M3 and M4) and a cytoplasm-facing re-entrant membrane loop (M2). Amino acids present within this loop control the permeation properties of the ion channel (Dingledine et al., 1999). AMPA-R and kainate-R mediate fast excitatory synaptic transmission (Chen et al., 2006), whereas NMDA-R mediate a slower phase of neurotransmission (Scannevin and Huganir, 2000).

6.4.1.2.1. AMPA receptors

AMPA-Rs are ubiquitously distributed throughout the CNS and are involved in fast excitatory synaptic transmission mediated by glutamate. They are composed of a family of four subunits called GluR1-4, which form tetrameric structures with homomeric or heteromeric assembly located in post-synaptic neuron (Chen et al., 2006). AMPA-Rs have a high permeability to Na^+ and a low one to Ca^{2+} . The Ca^{2+} permeability is dependent on the subunit composition, GluR2 subunit displaying very low permeability to Ca^{2+} due to a positively charged arginine at a critical site in the M2 domain of GluR2 instead of a glutamine (Dingledine et al., 1999). AMPA-Rs can present with further differences in properties depending on alternative splicing ('flip' or 'flop' subunits) that influence AMPA-R

desensitisation and allosteric modulatory properties (Bleakman and Lodge, 1998; Kew and Kemp, 2005). Interestingly, the CT (carboxyl terminal) of AMPA-R subunits presents some PDZ binding motifs (-SVKI for GluR2 and GluR3) allowing for interaction with scaffolding proteins and signalling molecules (Kim and Sheng, 2004). AMPA-R also play a role in the transition from silent to functional synapses.

6.4.1.2.2. Kainate receptors

Kainate receptors present a high expression level in the spinal cord, cerebellum, pyramidal neurons of the neocortex and the hippocampus (Bleakman and Lodge, 1998). They owe their name to kainite (Shinozaki and Konishi, 1970). Kainate receptors possess two subunit families, GluR5-7 and KA-1 and -2. They consist of a tetramer of homomeric or heteromeric assemblies. KA-1 and KA-2 subunits are unable to form functional homomeric receptors but instead have high affinity to kainate binding sites and can combine in heteromeric assemblies with GluR5-7 to form functional receptors (Bleakman and Lodge, 1998; Kew and Kemp, 2005). Each of the kainate receptor subunits comprises about 900 amino acids and can undergo alternative splicing and ribonucleic acid (RNA) editing, increasing the level of heterogeneity observed with these receptors (Chittajallu et al., 1999). Kainate receptors have been shown to be involved in excitatory transmission of the mossy fibres/CA3 pyramidal cell synapses of the hippocampus at a pre- and post-synaptic level (Darstein et al., 2003; Jaskolski et al., 2004). Kainate receptors also play a role in the strength of synaptic transmission and regulate the release of GABA and glutamate (Kew and Kemp, 2005).

6.4.1.2.3. NMDA receptors

The NMDA-R family is composed of seven subunits: NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B (Dingledine et al., 1999; Kew and Kemp, 2005). NMDA-R are tetrameric and typically contain two NR1 (GluN1) and two NR2 (A-D) (GluN2A-D) subunits. NMDA-R, unlike AMPA-R, is highly permeable to Ca^{2+} which enters the cell when its central ion channel is activated. The NMDA receptor is unique among all known neurotransmitter receptors in its requirement for the simultaneous binding of two different agonists for activation. In addition to the conventional agonist-binding site typically occupied by glutamate (NR2 subunit), the binding of glycine appears to be required for receptor activation (NR1 subunit). Because neither glycine nor glutamate acting alone can open this ion channel, they are referred to as co-agonists of the NMDA receptor (Dingledine et al., 1999; Erreger et al., 2004). At a neuronal resting membrane potential (-65mV), the NMDA-R ion channel is

blocked by extracellular magnesium ions (Mg^{2+}) which keep the receptor inactive. The AMPA-R-mediated depolarisation of post-synaptic neurons results in Mg^{2+} release from the NMDA-R ion channel leading to an activation of NMDA-R and Ca^{2+} influx and to a lesser extent Na^+ . The Ca^{2+} influx triggers a variety of intracellular signalling cascades including the activation of a Ca^{2+} /calmodulin complex, which stimulates nNOS, leading to the production of NO and the activation of guanylyl cyclase. The Mg^{2+} blocked under resting conditions accounts for the slower transmission mediated by NMDA-R compared to AMPA-R and kainate receptors (Kew and Kemp, 2005) (**Fig 1.8**).

6.4.2. NMDA-R antagonists

NMDA-R as a therapeutic strategy has been explored in fields of research including stroke, epilepsy, TBI, neuropathic pain, migraine, schizophrenia and major depression (Ikonomidou and Turski, 2002; Konradi and Heckers, 2003; Muir, 2006; Maeng and Zarate, 2007; Zhou et al., 2010; Lauritsen et al., 2016). NMDA-R antagonism can be achieved with receptor antagonists which block the ion channel such as ketamine, MK-801, phencyclidine (PCP) and memantine, [for review, (Abdallah et al., 2017)]. Ketamine, a common non-competitive antagonist of the NMDA-R has been used clinically primarily as an anaesthetic since the 1960s (Abdallah et al., 2017). Research has been focused on ketamine over the last 20 years as a single sub-anaesthetic dose leads to a rapid antidepressant effect in individuals with treatment-resistant depression. Ketamine is most commonly administered intravenously, but also subcutaneously, intramuscularly, transdermally, intranasally, intrarectally or orally (Abdallah et al., 2017). Ketamine's rapid antidepressant effects typically emerge about 4h after intravenous administration, well after the drug has been cleared from the bloodstream, and last for 1 to 2 weeks [for reviews, (Aan het Rot et al., 2012; Ibrahim et al., 2012)] and up to 28 days (Ibrahim et al., 2012b) in treatment-resistant patients (Berman et al., 2000; Zarate et al., 2006).

A single sub-anaesthetic dose of ketamine (0.5mg/kg) has been shown to produce a rapid and robust antidepressant response in patients within hours of administration (Berman et al., 2000; Zarate et al., 2006; Diazgranados et al., 2010). Two weeks after ketamine administration at 0.5 mg/kg, all depressive symptoms assessed by the Montgomery–Åsberg Depression Rating Scale (MADRS) were improved in ketamine-treated patients (Pennybaker et al., 2017). It has been demonstrated that supplementation of standard anaesthetics with

low-dose ketamine in depressive patients receiving ECT creates greater anti-depressive treatment effects (Li et al., 2010).

Animal studies have shown that a sub-anaesthetic dose of ketamine at 2.5mg/kg results in rapid, acute and sustained (2 weeks) antidepressant-like effects in rodent models of depression (Maeng et al., 2008). CUS, a stress model of depression, has been found to decrease the expression levels of synaptic proteins and spine number in the PFC. These effects can be rapidly reversed by ketamine administration (Li et al., 2011; Duman and Li, 2012) in addition to stress-related anhedonic and anxiogenic behaviours (Garcia et al., 2009; Li et al., 2011). Ketamine induces changes in PFC spines/synapses rapidly within 24 h of administration whereas conventional antidepressants require chronic administration.

In depression, post-mortem studies revealed a decrease in spine synapses number in the PLC (Kang et al. 2012), in line with studies of animal models of depression (**Fig 1.7**). It is proposed that there is a synaptic connectivity impairment in depression that can be reversed by ketamine treatment (Duman and Li, 2012). Ketamine-induction of synaptogenesis requires Mammalian target of rapamycin (mTOR) signalling and synaptic protein synthesis, suggesting that NMDA-R antagonists could have neurotrophic effects (Li et al. 2010). These synaptic changes are believed to be associated with stress-induced perturbations in the glutamatergic system (Abdallah et al., 2015). Stress leads to increased extracellular glutamate (Moghaddam, 1993), which, in excess, impairs synaptic strength and affects dendritic spine density (Massey et al. 2007). Ketamine has a duplicity of action; it can block the NMDA-R but also increase glutamate release. Although ketamine is known to act as an antagonist of post-synaptic NMDA-R, its mechanism targets other receptors. An important role for AMPA-Rs has been implicated in mediating the effects observed after ketamine administration. NBQX, an AMPA-R antagonist, was able to reduce ketamine's antidepressant-like effects (Maeng et al., 2008; Koike et al., 2011). Ketamine administration has been shown to regulate phosphorylation of the AMPA-R subunit GluR1 (Maeng et al., 2008). On the other hand, the activation of extrasynaptic NMDA-Rs reduces synaptic strength (Hardingham and Bading, 2010), whereas a blockade of extrasynaptic NMDA receptors by ketamine promotes synaptogenesis (Autry et al., 2011). In addition, recent research has provided insights into the stress-related synaptic dysfunction related to a reduction in BDNF and/or by inhibiting the mammalian target of rapamycin complex 1 (mTORC1) (Ota and Duman, 2013; Ronald S Duman et al., 2016). Indeed, ketamine has been shown to rapidly increase BDNF levels and

activate mTORC1 linked to an increase in synaptogenesis and connectivity (Li et al., 2010; Garcia et al., 2009). Blocking mTOR with rapamycin prevented ketamine-induced synaptogenesis and antidepressant-like effects (Li et al., 2010). To summarise, ketamine is proposed to act (1) by blocking NMDA receptors located on interneurons (Homayoun and Moghaddam, 2007), (2) by disinhibition of pyramidal neurons (3) leading to a glutamate excess (Moghaddam et al., 1997), (4) by activating AMPA-R stimulating BDNF release (Maeng et al., 2008) and (5) through the activation of the mTORC1 and BDNF pathways leading to (6) AMPA-R cycling and synaptogenesis (Garcia et al., 2009; Li et al., 2010). Another combined mechanism of ketamine involves the blockade of (1) extra-synaptic NMDA-R (Autry et al., 2011), (2) leading to disinhibition of eukaryotic elongation factor 2 (eEF2), (3) inducing BDNF translation, and (4) increasing protein synthesis, AMPA-R cycling and synaptogenesis (**Fig 1.7**).

Although a single administration of ketamine exerts antidepressant effects, there are some limitations to the use of ketamine suggesting it cannot be used long-term. NMDA-R activation is required for the normal function of the CNS, and blockade of NMDA-R function is therefore not desired. Clinical use of ketamine is currently limited due to its adverse effects. Ketamine has sedative and psychotomimetic side effects, and repeated exposure increases the risk of severe psychosis, dissociative episodes, and euphoria (Machado-Vieira et al., 2009). In addition, repeated administrations and/or add-on therapy are needed in order to maintain the effects of ketamine (Mathew et al., 2010; Ibrahim et al., 2012; Segmiller et al., 2013). For these reasons, targeting downstream of the NMDA-R (nNOS inhibitors or inhibitors of the PSD-95/nNOS interaction) would avoid non-specific NMDA-R antagonism side effect and specifically inhibit the NMDA-R/NO signalling pathway. Therefore, it could be a strategy to maintain the anti-depressive potential of NMDA-R antagonists without incurring the detrimental effects associated with their use.

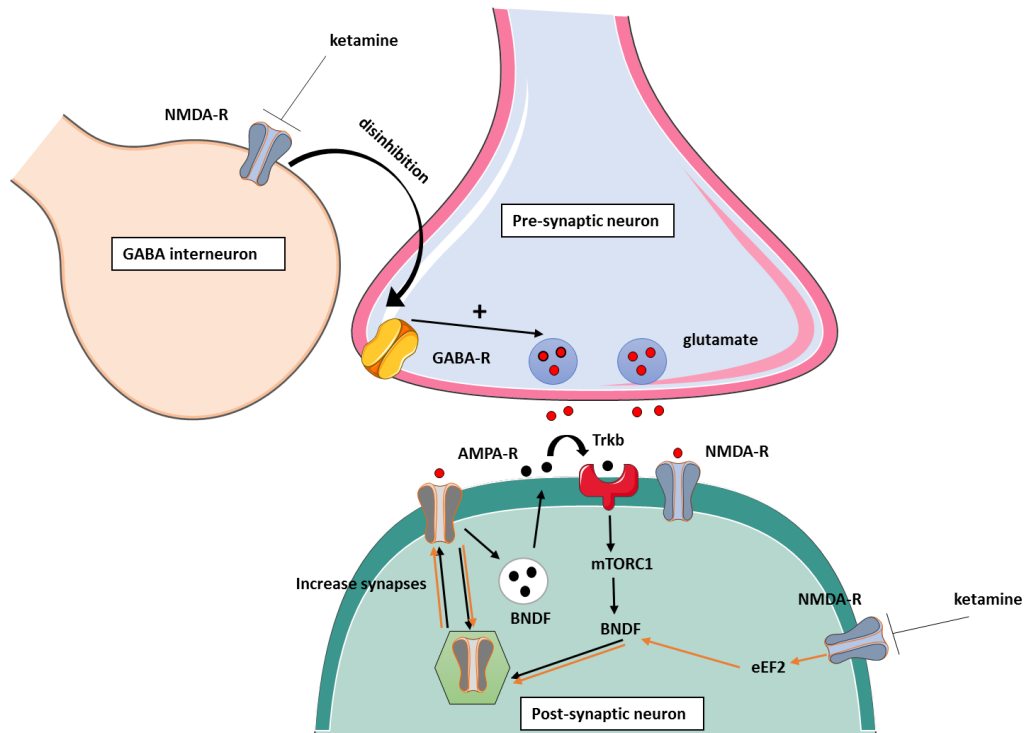


Figure 1.7: Proposed rapid mechanism of rapid antidepressive action of ketamine

Ketamine can act (black pathway) via a blockade of NMDA receptors located on GABA interneurons (1), disinhibiting pyramidal neurons (2), increasing glutamate release (3) and AMPA receptor activation (4), stimulating BDNF release (5), leading to activation of TrkB receptors (6), activating mTORC1 signaling, (7), inducing BDNF translation and protein synthesis (8), promoting AMPA-R subunit cycling, and synaptogenesis (9). In addition Ketamine can act directly on extrasynaptic NMDA receptors (orange pathway) (1), disinhibiting eEF2 (2), inducing BDNF translation and protein synthesis (3), AMPA-R subunit cycling, and synaptogenesis (4)

6.4.3. PSD-95/nNOS modulators

6.4.3.1. PSD-95

PSD is a membrane-associated mega-organelle located at the head of dendritic spines specialised in post-synaptic signal transduction and processing. Specifically PSD-95 is a scaffolding protein (Feng and Zhang, 2009). The PSD family of proteins are part of a larger class of scaffolding proteins known as the membrane-associated guanylate kinases (MAGUKs), including four PSD family members classified according to their molecular weight: PSD-95 (SAP-90), PSD-93 (Chapsyn-110), and synaptic-associated proteins 97 kDa and 102 kDa (SAP-97 and SAP-102, respectively). SAP-97 is found in the pre- and post-synaptic compartments, whereas PSD-95, PSD-93 and SAP-102 are found at the post-synaptic membrane of excitatory synapses (Kalia and Salter, 2003). The family of PSD proteins is composed of three PDZ domains, a SH3 (Src homology 3) domain and a GK (guanylate kinase) domain, which has lost its catalytic activity. PSD-95 interacts with several structural proteins and receptors, ion channels or pumps-transmembrane proteins, and

cytosolic proteins found at the synaptic terminal, specifically the NMDA-R GluN2B subunit and NOS via protein-protein interactions with binding to one or more of the three PDZ domains of PSD-95. Those interactions play a role in these proteins' precise assembly and spatial organisation as well as coupling receptors to downstream signalling events (Scannevin and Huganir, 2000) (**Fig 1.8**).

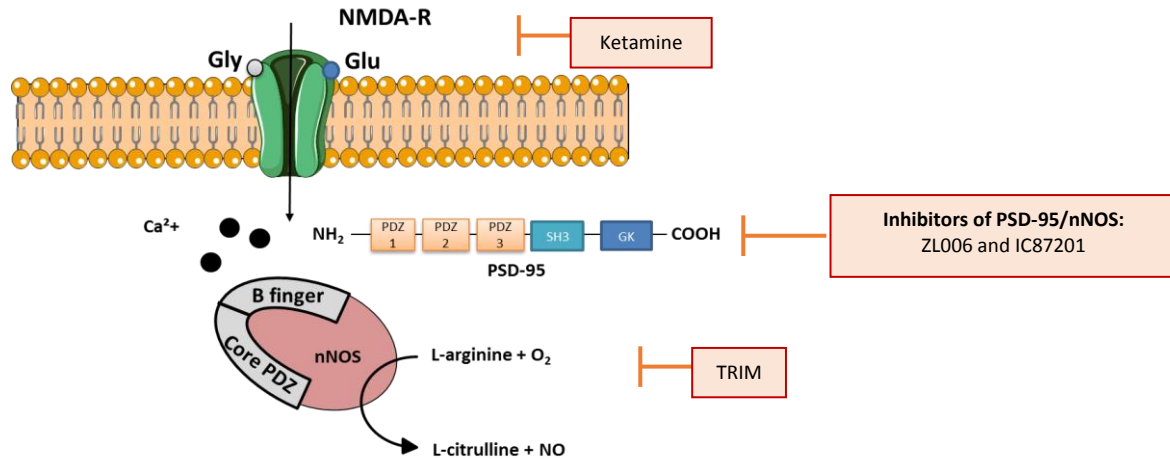


Figure 1.8: NMDA-R/PSD-95/nNOS complex

Glutamate-mediated NMDA-R stimulation triggers the influx of Ca^{2+} from the synapse into the post-synaptic neuron and by interaction between PSD-95 and nNOS via PDZ domains, leads to activation of NOS and production of NO and citrulline from arginine. NO leads to an activation of guanylyl cyclase and cGMP production. PSD-95 is made of three PDZ domains, a SH3 domain and a GK domain, which has lost its catalytic activity. PSD-95 interacts with NMDA-R via PDZ-1. PDZ-2 of PSD-95 interacts with the β -finger and core PDZ of nNOS. Ketamine acts as an NMDA-R antagonist, TRIM as a nNOS inhibitor and ZL006 and IC87201 as inhibitors of the interaction between PSD-95/nNOS.

6.4.3.2. NO signalling

Three NOS genes with distinct tissue localisation and properties are known: neuronal, inducible and endothelial nitric oxide synthase (nNOS, iNOS and eNOS) (nNOS [NOS1], iNOS [NOS2], and eNOS [NOS3], respectively). nNOS can be located either pre- or post-synaptically and is implicated in neural signalling, neurotoxicity, synaptic plasticity and the modulation of behaviour including learning and memory (Esplugues, 2002). NO was first characterised in the CNS as the intercellular messenger mediating the increase in cyclic GMP levels that follows the activation of glutamate receptors (Garthwaite et al., 1988). In addition, NO has been proposed as the retrograde messenger which co-ordinates the enhancement of both pre- and post-synaptic mechanisms involved in two forms of synaptic plasticity, LTP and LTD (Böhme et al., 1991; O'Dell et al., 1991) (Schuman and Madison, 1991).

Concerning the formation of NO, nNOS is closely coupled with Ca^{2+} - permeable NMDA-Rs at the PSD through their mutual PDZ binding motifs (Brenman et al., 1996). Following an

impulse mediated by glutamate from the pre-synaptic neuron, glutamate-mediated NMDA-R stimulation triggers the influx of Ca^{2+} from the synapse into the post-synaptic neuron (Moncada and Bolaños, 2006). Free cytosolic Ca^{2+} binds to calmodulin and activates nNOS, which forms NO and citrulline from arginine, leading to an activation of guanylyl cyclase and cGMP regulation (**Fig 1.8**).

6.4.3.3. nNOS inhibitors

The downstream inhibition of nNOS has also been investigated as an experimental approach to produce antidepressant effects avoiding the problems associated with direct inhibition of the NMDA-R. Ketamine can up-regulate the expression of nNOS mediating ketamine-induced neurotoxicity. Ketamine-induced neurotoxic effects were effectively blocked by 7-nitroindazole (7-NI). These data indicate a role for nitric oxide in the enhanced degeneration induced by ketamine in vitro and also suggest that blocking neuronal nitric oxide synthase (nNOS) may help reduce the risk of ketamine in pediatrics (Wang et al., 2008).

Although L-arginine (L-arg)-derived NOS inhibitors cannot be considered isoform-selective, of these inhibitors N^{ω} -nitro-L-arginine (L-NA) has been reported to be the most selective in inhibiting brain NO synthesis (Moncada et al., 1991). 1-[2-(trifluoromethyl)phenyl]imidazole (TRIM) is a selective inhibitor of nNOS with weak inhibition against eNOS (Handy and Moore, 1998; Haga et al., 2003). Preclinical studies have reported antidepressant-like effects of NOS inhibitors in a range of antidepressant-sensitive behavioural tests in rodents (Wegener and Volke, 2010; Sherwin et al., 2017a,b). N^{G} -nitro-L-arginine (L-NNA), N^{G} -monomethyl-L-arginine (L-NMMA), N^{G} -nitro-L-arginine methylester (L-NAME) and TRIM demonstrated a similar efficacy to the conventional tricyclic antidepressant imipramine in the mouse forced swim test (FST) (Harkin et al., 1999; Volke et al., 2003). L-NA and TRIM reduced hyperactivity in the open field in a similar fashion to imipramine treatment in the olfactory bulbectomised rat model of depression (Gigliucci et al., 2014).

By contrast to NMDA receptor antagonists, NOS inhibitors don't produce effects on locomotor behaviour in the open-field (OF) (Harkin et al., 1999; Harkin et al., 2004). In other preclinical studies, nNOS deletion or treatment with the nNOS inhibitors 7-nitroindazole or TRIM prevented CMS-induced depression-related behavioural changes in mice and produced a faster onset of action compared to the traditional antidepressant fluoxetine (Mutlu et al., 2009; Zhou et al., 2007). Interestingly, mice exposed to CMS over-expressed hippocampal nNOS and showed impaired neurogenesis in the hippocampus (Zhou et al., 2007). CMS-

induced behavioural deficit as measured by increased immobility in the tail suspension test (TST) and impairment in hippocampal neurogenesis were reversed in mice lacking the nNOS gene or mice treated with 7-NI. Moreover, disrupting hippocampal neurogenesis blocked the antidepressant effects of 7-NI in this model. These results suggest that nNOS over-expression and reduced neurogenesis in the hippocampus are associated with stress-induced depression and that inhibition of nNOS signalling in the brain represents a novel approach to antidepressant treatment.

6.4.3.4. Inhibition of PSD-95/nNOS

As NMDA-R antagonists possess antidepressant properties but are currently limited and cannot be used long-term due to their side effects, targeting downstream of the receptor such as nNOS may represent a target for antidepressant activity. However, highly selective inhibitors of nNOS have been difficult to develop. In this regard, the selective approach of targeting PSD-95/nNOS may be used to further test for a novel glutamatergic NO-based treatment for depression.

Acting downstream of the NMDA-R, small molecular weight compounds have been developed which inhibit the PSD-95/nNOS interaction (Doucet et al., 2012) (**Fig 1.8**). 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 IC₅₀ of 31µM without inhibiting nNOS catalytic activity (Florio et al., 2009). In a mouse model of NMDA-induced thermal hyperalgesia, intrathecal administration of 1pM of IC87201 and following an intraperitoneal injection (0.1mg/kg) of IC87201 resulted in antinociceptive effects. IC87201 also abolishes mechanical allodynia after intrathecal (at 50 and 100nM dose) and intraperitoneal (2mg/kg) administration in a rat model of sciatic nerve constriction (Lee et al., 2015). IC87201 has been shown to inhibit pain behaviour and formalin-induced spinal FOS protein expression without producing motor ataxia observed following treatment with the NMDAR antagonist MK-801 in rats (Carey et al., 2017). These results indicate that IC87201 is capable of crossing the BBB and could be a promising drug to investigate in the treatment of pain (Florio et al., 2009).

Another small molecular weight compound was later characterised, ZL006 (4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid) (Zhou et al., 2010). 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, is

structurally required for the interaction of nNOS and the PDZ-2 of PSD-95. This is supported by the fact that the mutation of Arg-121 has been shown to destabilise the nNOS β -finger, preventing nNOS-PDZ from binding to PSD-95-PDZ-2, which shows 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 the Arg-121 nNOS β finger, and a hydrophobic bond between the hydrophobic ring of ZL006 and Leu-107 or Phe-111 of nNOS, interrupting the conformational change of the nNOS PDZ domain (Zhou et al., 2010). This compound has been shown to inhibit the 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. A dose of 1.5 mg/kg has shown preclinical efficacy in a middle cerebral artery occlusion model of middle cerebral artery occlusion (MCAO) model of stroke in mice, without affecting nNOS expression or catalytic activity or NMDA-R functions. In a recent study, ZL006 has been found to protect spinal cord neurons against ischemia-induced oxidative stress (Liu, S.G et al., 2017). Interestingly, using a carrier (ZL006-loaded-nanocarrier based on liposomes) that can penetrate the BBB and subsequently targeting the ischemia area in brain, ZL006 ameliorated the infarct volume and the neurological deficit of MCAO rats (Zhao et al., 2016). Recent data also indicated the utility of these small molecules as novel analgesics, and showed that both ZL006 and IC87201 had antinociceptive effects in animal models of inflammatory and neuropathic pain (Lee et al., 2015). ZL006 was able to inhibit pain behaviour and formalin-induced spinal FOS protein expression (Carey et al., 2017). In another study, formalin-induced conditioned place avoidance (F-CPA) was reduced after intra-BNST injection of the PSD-95–nNOS interaction inhibitor ZL006, as well as the NMDA-R antagonist MK-801 and the nNOS inhibitor NG-propyl-L-arginine (NPLA) (Deyama et al., 2017). Both IC87201 and ZL006 have also demonstrated neuroprotective effects against 1-methyl-4-phenyl-pyridinium (MPP⁺)-induced neuronal injury and apoptosis in an *in vitro* model of Parkinson's disease (Hu et al., 2014).

Further research confirmed the efficacy of ZL006 in MCAO models and demonstrated that dissociating PSD-95 from nNOS using ZL006 (1.5mg/kg/d, i.v.) enhanced neuronal survival, promoted the migration of new neurons into the injury area and fostered neurite outgrowth in newborn neurons and dendritic spine formation in mature neurons in the ischaemic brain (Luo et al., 2014). In addition, ZL006 has also been shown to promote the migration and differentiation of transplanted neural stem cells in male rats after a stroke (Wang et al., 2017).

Previous *in vitro* research from our laboratory showed that PSD-95–nNOS interaction inhibitors (IC87201 and ZL006) appear to exert similar effects on neuronal complexity to those of NMDA-R antagonists and nNOS inhibitors (Doucet et al., 2015). Indeed, treatment with the NMDA-R antagonist ketamine and MK-801 (10nM) in addition to TRIM (100nM) counteracted the NMDA/glycine-induced reduction in neurite outgrowth. Similar effects were obtained with IC87201 (10 and 100nM) and ZL006 (10 and 100nM) (M.V. Doucet et al., 2015). Furthermore, these data support the hypothesis that targeting the NMDA-R/PSD-95/nNOS interaction downstream of NMDA-R promotes neurotrophic effects by preventing neurite atrophy in response to excessive glutamatergic stimulation.

In addition, IC87201 (1-2mg/kg) and ZL006 (10mg/kg) produced antidepressant-like responses in the FST and TST following a single administration in mice. By contrast to the tricyclic antidepressant imipramine (25mg/kg), the effects are not observed 1h following drug administration but are apparent 24 and 72 h later. Furthermore prior exposure to the TST or FST is required in order to observe antidepressant-related activity. Similar delayed and sustained antidepressant-like effects were observed following TRIM (50mg/kg) and ketamine (30mg/kg) in the TST (Doucet et al. 2013). Similar antidepressant-related properties for ZL006 were observed in the FST in rats (Sherwin et al., 2017b).

Together, these findings indicate that PSD-95–nNOS interaction inhibitors exert effects equivalent to those of NMDA-R antagonists and nNOS inhibitors, and could therefore be an attractive target for treating ischemia, pain or neurodegenerative disease. Moreover, behavioural measures associated with NMDA-R antagonism and nNOS inhibitors have significant side effects, such as spatial and episodic (source) memory, aggressive behaviour, reduced locomotion, and decreased motor coordination (Dzoljic et al., 1997; Kemp and McKernan, 2002; Volke et al., 2003; Zhou et al., 2010; Doucet et al., 2013; Smith et al., 2016), not present with PSD-95/nNOS interaction inhibitors (Doucet et al., 2013; Lee et al., 2015; Smith et al., 2016). These results indicate that ZL006 disrupts PSD-95/nNOS without causing adverse effects and support its potential for drug development (Doucet et al., 2012).

6.5. Effect of antidepressant treatment on neuronal plasticity

Recently, particular interest has been given to the effects of classical antidepressants on adult hippocampal neurogenesis and neuronal plasticity. A number of these drugs have been shown to reverse the effects of chronic stress on behaviour accompanied by increased dendritic arborisation in the CA3 and DG of the hippocampus as well as the PFC [for reviews, (Duman

et al., 2016; McEwen et al., 2016)]. Several hypotheses have been put forward for how these drugs may reverse the effect of stress on neuronal complexity (Duman and Duman, 2015; McEwen et al., 2016). Interestingly, chronic intraperitoneal injection of fluoxetine (10mg/kg), imipramine (10-15mg/kg), desipramine (10mg/kg) and two putative antidepressants, CP 156,526, a type 1 corticotropin-releasing hormone receptor antagonist (20mg/kg) and SSR 149415, a type 1b arginine vasopressin receptor antagonist (30mg/kg), have been shown to reverse dendritic atrophy in the hippocampus following chronic stress or corticosterone administration (Bessa et al., 2008; Hajszan et al., 2009; Fenton et al., 2015) in the PFC (Bessa et al., 2008; Nava et al. 2017) and lithium (4-5mg/kg/day) reversed chronic stress-induced dendritic hypertrophy in the amygdala (Johnson et al., 2009) **(for details refer to table 1.5).**

Table 1.5: Effect of antidepressant drugs on neuronal complexity

Structure	Substructure	Species	Treatment	Neuronal complexity	Reference
Hippocampus	Granule cells and CA3 (Pyramidal cells)	Male Wistar rats	Chronic Fluoxetine, imipramine, CP 156,526, or SSR 149415	Dendritic atrophy reversal (only apical for CA3 cells) from chronic mild stress	(Bessa et al., 2008)
	Reelin+ cells (Subgranular cells)	Adult male Long–Evans rats	Chronic Imipramine	Reversal of loss in number and complexity of immature neurons from chronic corticosterone injections	(Fenton et al., 2015)
	CA1 and CA3 and dentate gyrus (Pyramidal and granular cells)	Adult male Sprague-Dawley rats	Chronic Desipramine	Loss of synapse reversal from learned helplessness	(Hajszan et al., 2009)
Prefrontal	Layer II/III PFC (Pyramidal neurons)	Male Wistar rats	Chronic Imipramine, CP 156,526, SSR 149415	Apical dendritic atrophy attenuation from chronic mild stress	(Bessa et al., 2008)
	Prelimbic regions	Male Sprague-Dawley rats	Chronic Desipramine	Apical dendritic atrophy prevention (1 day and 14 days after stress) from foot-shock stress	(Nava et al., 2017)
Amygdala	BLA (Pyramidal neurons)	Male Sprague–Dawley rats	Chronic Lithium	Prevention of chronic stress-induced increase in dendritic branching by reducing dendritic length	(Johnson et al., 2009)

At another level, antidepressant drugs can reverse the effect of stress on spine density (Duman and Duman, 2015; McEwen et al., 2016) (**for details refer to table 1.6**). Fluoxetine (10mg/kg), combined with brexiniprazole, D₂ dopamine partial agonist (0.1mg/kg) or alone, attenuates the decrease in spine density caused by stress in the hippocampus, the PFC or the nucleus accumbens (Ma et al., 2016; Hajszan et al., 2005; Bessa et al., 2008). Imipramine (10-15mg/kg) increased the number of spines in the PFC following chronic stress (Bessa et al., 2008). In addition, a single dose of NMDA-R antagonist (ketamine or R-ketamine, 10, 20mg/kg) also appears to be sufficient to protect spines against stress in the hippocampus and the PFC in mice (Yang et al., 2015; Dong et al., 2017). Interestingly, ketamine by its antagonism of NMDA-R appears to allow spines to maintain their current morphology,

whereas NMDA-R activation has been shown to cause spines to collapse (Hering and Sheng, 2001).

Drugs in development have also been shown to prevent the effect of stress on spines. For example, MGS0039, a metabotropic glutamate receptor antagonist (1mg/kg), GLYX-13, a monoclonal antibody that binds and modulates the NMDA-R (3mg/kg) and Scopolamine, a muscarinic receptor antagonist (25µg/kg), increase the number of spines in the PLC following stress (Voleti et al., 2013; Burgdorf et al., 2015; Dong et al., 2017; Liu R.J, et al., 2017). Similar results were obtained in the hippocampus and PFC with CP 156,526 (20mg/kg) and SSR 149415 (30mg/kg) (Bessa et al., 2008; Ma et al., 2016). To conclude, antidepressant drugs appear to allow spines to remain in their current morphology in the face of stress-related alterations.

Table 1.6: Effect of antidepressant drugs on spine density

Structure	Substructure	Species	Treatment	Spines	Reference
Hippocampus	Dentate gyrus and CA3(Pyramidal neurons)	Male adult C57BL/6 mice	Acute combination of fluoxetine and brexipiprazole	Attenuation of decreased spine density from social defeat stress	(Ma et al., 2016)
		Male adult C57BL/6 mice	Acute ketamine (single-dose), MGS0039 (single-dose)	Attenuation of reduction spine density from social defeat stress	(Dong et al., 2017)
		Male adult C57BL/6 mice	Acute R-ketamine (single dose)	Attenuation of reduction spine density from learned helplessness	(Yang et al., 2015)
	CA1 and CA3 (pyramidal cells)	Ovariectomized female rats	Chronic fluoxetine	5 days injection induces increase spine density in CA1 field with an effect CA3 after 2 weeks of treatment in learned helplessness model	(Hajszan et al., 2005)
	Dentate gyrus (Pyramidal neurons)	Adult male (2–3 month old) Sprague-Dawley (SD) rats	Acute GLYX-13 (Rapastinel)	Enhancement of synaptic plasticity processes & increase in number of mature dendritic spines following FST	(Burgdorf et al., 2015)
	layer V of anterior cingulate and prelimbic mPFC	Male Sprague–Dawley rats weighing 150–250 g	Acute ketamine	Reversal of apical and basal spine (mushrooms) deficit due to CUS, increase	(Li et al., 2010, 2011)

Prefrontal cortex					in total spine number		
	Prelimbic regions of the PFC	Male adult C57BL/6 mice		Acute combination of fluoxetine and brexipiprazole	Attenuation of decreased spine density from social defeat stress	(Ma et al., 2016)	
		Male adult C57BL/6 mice		Acute ketamine, MGS0039	Attenuation of reduction spine density from social defeat stress	(Dong et al., 2017)	
		Male adult C57BL/6 mice		Acute R-ketamine	Attenuation of reduction spine density from social defeat stress	(Yang et al., 2015)	
	Layer V in the mPFC (pyramidal neurons)	Male Sprague Dawley rats (Charles River Laboratories)		Acute GLYX-13 (Rapastinel)	Rapid increase of number and function of spine synapses in the apical dendritic tuft following FST	(R.-J. Liu et al., 2017)	
		Adult male (2–3 month old) Sprague-Dawley (SD) rats			Increase of the proportion of mature spines, and the density of stubby spines following FST	(Burgdorf et al., 2015)	
		Male Sprague-Dawley rats weighing 175–250 g		Acute scopolamine	Increase in spine synapses following FST	(Voleti et al., 2013)	
		Male Wistar rats		Chronic fluoxetine, imipramine, CP 156,526, or SSR 149415	Spine density recovery due to chronic mild stress	(Bessa et al., 2008)	
	Nucleus accumbens	(Core and Shell neurons)	Male adult C57BL/6 mice	Acute combination of fluoxetine and brexipiprazole	Attenuation of decreased spine density from social defeat stress	(Ma et al., 2016)	

7. Astroglia and neuronal interactions

7.1. Glial cells

The CNS is made up of several cell types including neurons, which are responsible for the coordination of all bodily functions, and glia. Glial cells have been classified into four major types (astrocytes, oligodendrocytes, microglia and ependymal cells). Microglia are derived from hematopoietic stem cells in mesodermal tissue, and astrocytes and oligodendrocytes from the ectodermal tissue. Ependymal cells, a type of neuronal support cells are derived

from a layer of embryonic tissue known as neuroectoderm and form the epithelial lining of the ventricles (cavities) in the brain and the central canal of the spinal cord

Astrocytes and oligodendrocytes play a major role in neurons' support and structural integrity, whereas microglia are implicated in immune function. In particular, astrocytes interact directly with neurons and blood vessels via astrocytic end-feet and oligodendrocytes are important for supporting neurons by producing myelin. Glial cells have diverse roles in the brain in relation to, e.g., the regulation of the regional blood flow and energy metabolism, immune defence, amino-acid neurotransmitter clearance, neurotrophin production and the modulation of synaptic connections and adult neurogenesis (**Fig 1.9**) (Czéh and Di Benedetto, 2013).

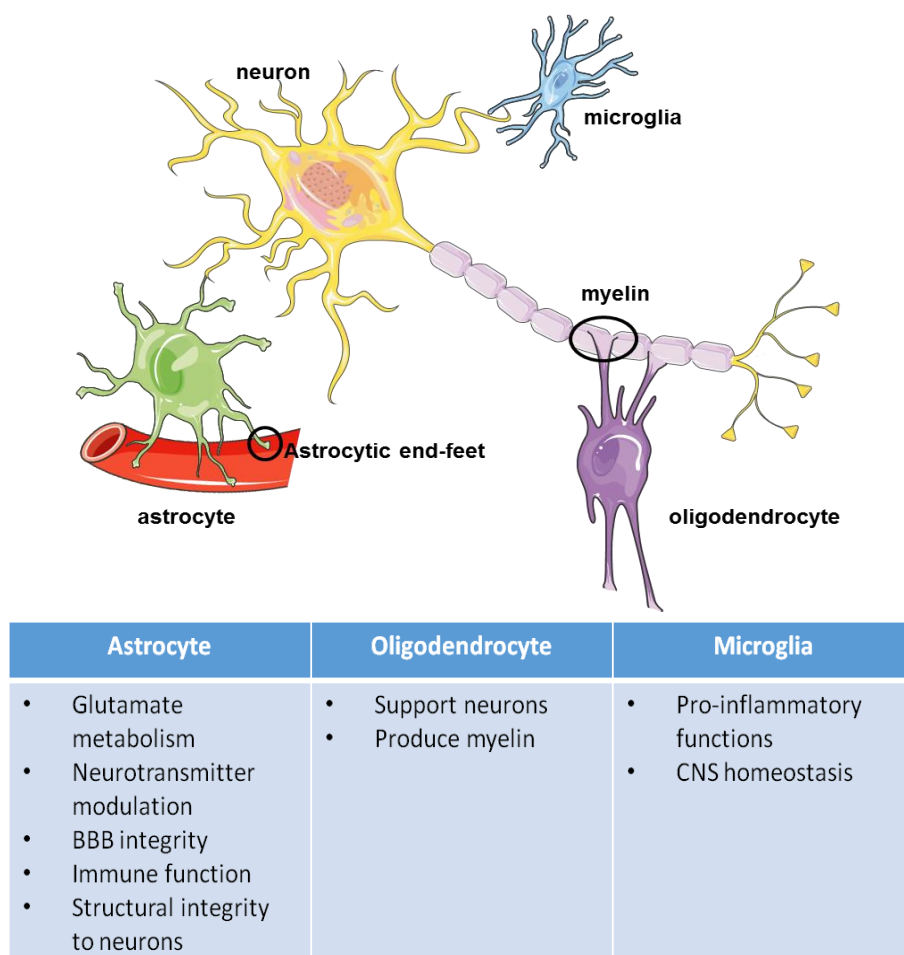


Figure 1.9: A summary of the various functions of glial cells

Glial cells exhibit a range of functions. Astrocytes are the most abundant and prevalent cells of the CNS. They play a major role in neurotransmission modulation, immune regulation, maintenance of the BBB and integrity of neurons. Oligodendrocytes are important for supporting neurons by producing myelin, while microglia are primarily concerned with immunosurveillance and regulation in the brain.

Astrocytes are classified into two main categories: fibrous and protoplasmic. Fibrous astrocytes are found particularly in the white matter while protoplasmic astrocytes are located in the grey matter (**Fig 1.10**). Astrocytes exert a homeostatic function by regulating neurotransmitters in the brain in addition to the degradation of excitotoxic neurotransmitters. Astrocytes also play a role in the inflammatory response and are also a key component of the blood brain barrier (BBB) by surrounding blood vessels (Koehler et al., 2009) that prevent the passage of molecules, ions and cells into the brain parenchyma from the bloodstream as well as maintaining cerebrovascular tone.

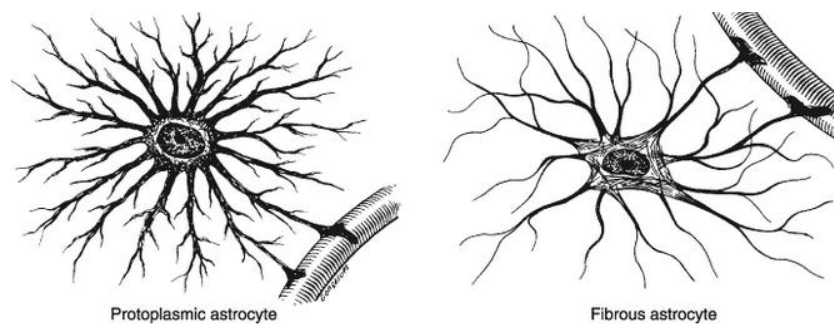


Figure 1.10. Different types of astrocytes

Protoplasmic and fibrous astrocytes not only differ by their location (Grey matter for protoplasmic and white matter for fibrous astrocytes) but also in their morphology. Protoplasmic astrocytes presents a higher branching compared to fibrous astrocytes. *Ganong Wf, Review of medical physiology, 22nd edition*

Immunohistochemical techniques that enable the detection of astrocytic specific molecular markers at the single-cell level are essential tools for identifying and characterizing cells in healthy and pathological tissue. Expression of glial fibrillary acid protein (GFAP) has become a prototypical marker for immunohistochemical identification of astrocytes (Sofroniew and Vinters, 2010a). GFAP expression can be regarded as a sensitive and reliable marker that labels most, if not all, reactive astrocytes that are responding to CNS injuries.

However, GFAP is not an absolute marker of all non-reactive astrocytes and is often not immunohistochemically detectable in astrocytes in healthy CNS tissue. Other molecular markers that have been used for immunohistochemical identification of astrocytes and reactive astrocytes include the glutamine synthetase (GS) and S100 β (S100b) but these molecules are not entirely exclusive to astrocytes. One other marker, the protein Aldh1L1 may also provide a sensitive chemical marker for most if not all astrocytes in healthy conditions (Sofroniew and Vinters, 2010a).

7.2. Gliogenesis

Astrocytes at different stages of embryonic and post-natal development are morphologically different. While there are no specific markers to characterise the different stages of astrocytogenesis, it seems that astrogliosis occurs in four stages: (1) the radial glial progenitor in the ventricular zone (VZ) generates a proliferating intermediate progenitor that (2) migrates in the sub-ventricular zone (SVZ) and further. The mechanism of migration of astrocytes isn't well understood and subject to interpretation. They could travel along restricted radial trajectories (Gray and Sanes, 1991), or employ multimodal migration to migrate to various regions of the CNS, as suggested by numerous transplant experiments (Zhou et al., 1990; Jacobsen and Miller, 2003). (3) It leads to the formation of (3) immature astrocytes and (4) mature adult astrocytes. Further, radial glia/stem cells in the VZ are involved in two distinct processes (**Fig 1.11**). First, during the embryonic period, they are involved in neurogenesis and switch to become gliogenic in the cortex at around E16-18 (Deneen et al., 2006) to give rise to proliferating astrocyte and oligodendrocyte precursors (Molofsky et al., 2012). Second, during the first three post-natal weeks astrocyte proliferates 6- to 8-fold in the murine brain (Bandeira et al., 2009).

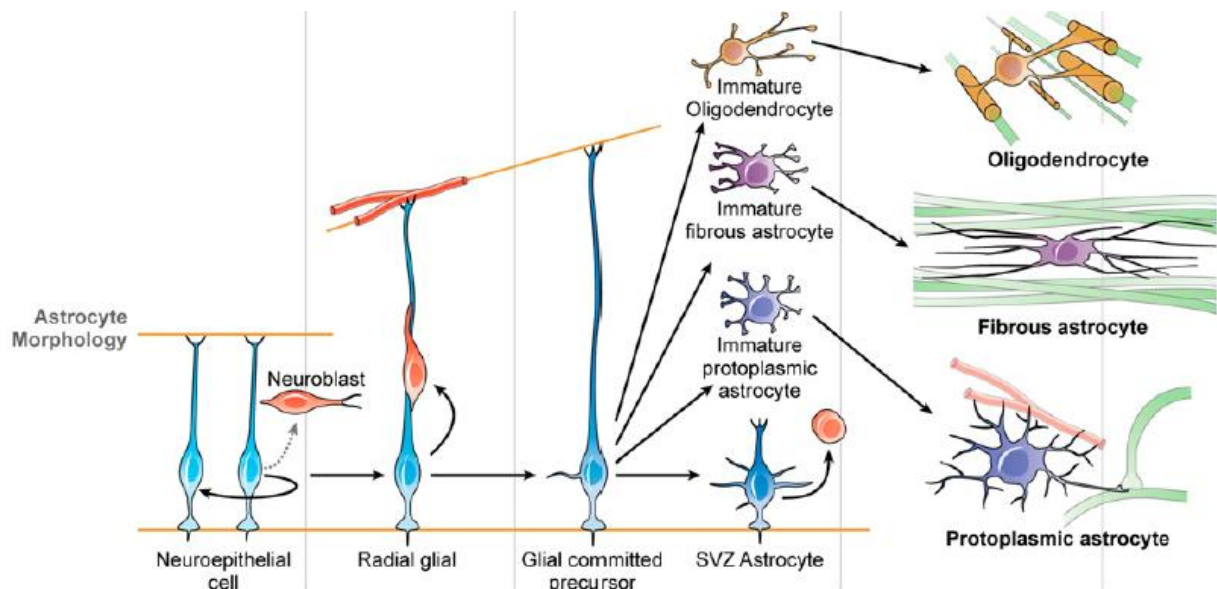


Figure 1.11: Gliogenesis

Neuroepithelial cells give rise to radial glia. In early stages, they are involved in neuronal proliferation but then become glial-committed. After proliferation, these cells give rise to immature oligodendrocyte, fibrous and protoplasmic astrocytes in the subventricular zone (SVZ) until maturation. Adapted from Molosky et al. (2012).

7.3. Trophic support of astrocytes

It appears that astrocytes in the rat's cortex represent about 90% of cortical tissue volume, the remaining 10% consisting of neuronal cell bodies and blood vessels (Chao et al., 2002).

Astrocytes are functionally linked to neurons via astrocytic end-feet. A single astrocyte in the human cortex may regulate the function of several neurons (Koehler et al., 2009). Among glial cells, astrocytes are the most versatile. Astrocytes can actively modulate synaptic transmission through their formation of a tripartite synapse, whereby one astrocyte is in close proximity to a neuronal synapse, which is made up of a pre- and a post-synaptic neuron (Haydon, 2001).

Astrocytes are known to actively secrete trophic factors into their environment, which promote neuronal growth and development (Barres, 2008; Allen, 2013), including BDNF, NGF- β , FGF-2, GDNF, and IL-6 (Day et al., 2014; Takemoto et al., 2015). Media derived from astrocytes – termed conditioned media (CM) which would contain soluble factors released by astrocytes – has been shown to enhance the branching of dendrites (Previtera et al., 2010) and to be sufficient to protect neurons against glutamate-induced apoptotic cell death in rat spinal cord neurons (Lu et al., 2015), methylmercury (Takemoto et al., 2015) and corticosterone-induced damage in primary culture of hippocampal neurons (Zhu et al., 2006), and partially attenuate excessive ROS and reactive nitrogen species (RNS) (Tanaka et al., 1999). Astrocytes also 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).

Astrocytes have been shown to strongly increase the number and efficacy of synapses *in vitro*, and are known to be crucial for the formation of fully functional synapses (Ullian et al., 2004). Glial-derived CM (soluble factors derived from glia) is capable of promoting synapse formation and maturation (Nägler et al., 2001). Astrocytes actively secrete molecules which promote synaptogenesis, including hevin (Allen, 2013), cholesterol complexed to apolipoprotein-E containing lipoproteins (Mauch et al., 2001) as well as thrombospondins (TSPs) (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 (Christopherson et al., 2005). Astrocytes have also been shown to mediate synaptic strength through the production of glial-derived TNF α (Beattie et al., 2002).

A diverse range of receptors and transporters for all the major neurotransmitter systems (glutamate, GABA, adrenaline, serotonin, dopamine, acetylcholine, and purine) are present in these cells which allow them to exert a homeostatic function in the brain in addition to the degradation of excitotoxic neurotransmitters. Astrocytes also play a role in the inflammatory response as they express receptors for cytokines, neuropeptides and corticosteroids, allowing them to detect and take action to prevent excessive inflammatory responses by releasing anti-inflammatory mediators (Chen et al., 2006; Rajkowska and Miguel-Hidalgo, 2007; McNally et al., 2008; Pav et al., 2008). In addition, astrocytes are also a key component of the blood brain barrier (BBB) by surrounding blood vessels (Koehler et al., 2009), and prevent the passage of molecules, ions and cells into the brain parenchyma from the bloodstream while maintaining the cerebrovascular tone. Furthermore, it has been suggested that astrocytes functionally connect with oligodendrocytes, the cells responsible for the myelination of axons in the CNS, which enhances neural efficiency of communication. Oligodendrocytes are coupled with astrocytic cells and the breakdown of this connection can contribute, for example, to the development of demyelination neuropathies (Rash et al., 2001; Orthmann-Murphy et al., 2008). Overall, astrocytes are involved in several key mechanisms in the brain and as such could play a major role in several debilitating CNS disorders.

7.4. Dysfunctional astroglia/neuronal interaction

Astrocyte activation plays a central role in the cellular response to brain insults like trauma, infections, stroke, tumorigenesis, and neurodegeneration. However, chronic astrocyte activation can also interfere with normal brain function. Astrocytes activation, referred as “reactive gliosis” is characterised by changes in the expression of many genes and characteristic morphological hallmarks. Reactive astrocytes present a hypertrophy of cellular processes and upregulation of the glial fibrillary acidic protein (GFAP), which is the main constituent of the intermediate filament system of adult astrocytes (Pekny et al., 2014). The exact mechanism by which astrocytes become dysfunctional remains unknown. However, the glial glutamate transporters seems to represent a spanning phenomenon common to many pathological conditions increasing extracellular glutamate concentrations leading to excitotoxic neuronal damage (Seifert et al., 2006a).

Soluble factors secreted from dysfunctional astrocytes or lack of soluble neuroprotective factors from the loss of healthy astrocytes may negatively affect synapse formation and development with many CNS disorders, including schizophrenia (Harrison, 2004), MDD

(Cotter et al., 2001; Rajkowska and Miguel-Hidalgo, 2007) and Alzheimer's disease (Shankar et al., 2007). 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 (McKeon et al., 1991; Snow et al., 1990; Smith-Thomas et al., 1994). In addition, a previous study demonstrated that cultured neurons exposed to CM from dysfunctional ammonia-treated astrocytes decreased the number of synapses (Jayakumar et al., 2014).

It is important to consider whether stress and its resultant HPA axis activation could induce astrocytic dysfunction, further identifying astrocytic dysfunction as a possible mechanism of depression. The glial fibrillary acid protein (GFAP) helps to maintain astrocytes' strength and shape, playing a role in astrocyte-neuronal communication. However, stress causes a reduction of astrocytic markers such as GFAP and changes in astrocytic morphology in the regions of the brain that are predominantly affected in depression including the PFC and the hippocampus (Nestler et al., 2002).

The impaired glutamate homeostasis observed in MDD patients may be related to the reduced astrocytic packing density detected from post-mortem studies on MDD brain samples. Furthermore, reduced expression of GLAST and GLT-1 occur in anterior cingulate (Choudary et al., 2005), dorsolateral prefrontal (Choudary et al., 2005; Klempan et al., 2007) and orbitofrontal cortex (Miguel-Hidalgo et al., 2010), and in locus coeruleus (Bernard et al., 2010; Chandley et al., 2013) and hippocampus (Medina et al., n.d.) of subjects diagnosed with MDD.

Treatment with glucocorticoids reduces GFAP mRNA expression in the hippocampus of rats probably through the type II glucocorticoid receptor (Simard and Nedergaard, 2004).

In addition, chronic psychosocial stress has been found to induce a marked reduction of astrocytic somal volume and GFAP expression in tree shrews (*Tupaia Belangeri*), which was reversed by fluoxetine treatment (Czeh et al., 2005). This effect seems to follow the trend of reduced glial volume and GFAP expression in post-mortem studies of depressed patients' brains in the PFC and the hippocampus (Rajkowska et al., 1999; Rajkowska and Stockmeier, 2013).

As astrocytes are important for the viability and maturation of stem-cell-derived neurons, neurons critically depend on their intrinsic protective and supportive properties. Dysfunction of astrocytes leads to impaired neuronal functionality and viability (Liu B., et al., 2017).

After brain ischemia, astrocytes presented a decrease in glutamate transport activity, GFAP, s100 calcium binding protein b (S100b) and GLT-1 immunoreactivity, leading to the degeneration of CA1 neurons, indicating that the vulnerability of CA1 neurons may be secondary to selective ischemia-induced astrocytic dysfunction (Ouyang et al., 2007). Reactive glial ablation with the antiviral agent ganciclovir treatment in mice leads to a decrease in GFAP immunoreactivity and a substantial neuronal degeneration attenuated by chronic glutamate receptor blockade (Bush et al., 1999).

In another study, reactive A1 astrocytes (inflammatory astrocytes), induced by activated neuroinflammatory microglia, lost the ability to promote neuronal survival, outgrowth, synaptogenesis and phagocytosis, and induced neuronal death. However, by blocking A1 astrocytes with weekly injection of neutralising antibodies to interleukin 1 α (IL-1 α), TNF α and C1q or with a triple (*Il-1 α ^{-/-}TNF α ^{-/-}C1q^{-/-}*) knock-out mice which fail to generate A1 astrocytes, the death of axotomised neurons was prevented (Liddel et al., 2017).

7.5. Evidence of astrocytic abnormalities in depression

The recent literature has been focusing on the putative role of glial cells, specifically astrocytes, in the pathology of MDD and bipolar disorder. There is increasing evidence to suggest that a disruption in normal astrocyte structure and function may play an important role in these disorders. A number of post-mortem studies in MDD patients have shown that there are significant reductions in glial density in the fronto-limbic areas, especially the PFC and mPFC, the dorsolateral and orbito-frontal cortex (Rajkowska and Miguel-Hidalgo, 2007), the subgenual cingulate cortex (Williams et al., 2012), the amygdala (Bowley et al., 2002) and the left hippocampus (Cobb et al., 2016). However, another study showed that there is an increase in glia density in all hippocampal subfields and the DG, with a decrease in pyramidal neuron soma size (Stockmeier et al., 2004). These results could explain the decreased hippocampal volume reported in MDD patients, as an increase in density might be associated with a decrease of glial branching, a consequence of a disconnection from neurons (Rajkowska and Miguel-Hidalgo, 2007).

Glutamate is the major excitatory neurotransmitter in the brain. Its synaptic availability is regulated via the excitatory amino acid transporter (EAATs) located on the astrocytic end-feet. Once in the astrocyte, glutamate is converted by the astrocytic enzyme GS into glutamine (Rothman et al., 2012). Glutamine is then released from the astrocyte and can be taken back up by neurons to act as a substrate for further glutamate synthesis (Martinez-Hernandez et al., 1977). In pathological conditions, increased levels of synaptic glutamate may exert excitotoxic effects on neurons, triggering either rapid or delayed neurotoxicity [for review, (Sanacora et al., 2008)] (**Fig 1.12**). As a consequence of their prominent role in modulating the levels of synaptic glutamate, astrocytes appear to play a key role in neuronal protection by regulating extracellular levels of glutamate (Seifert et al., 2006b).

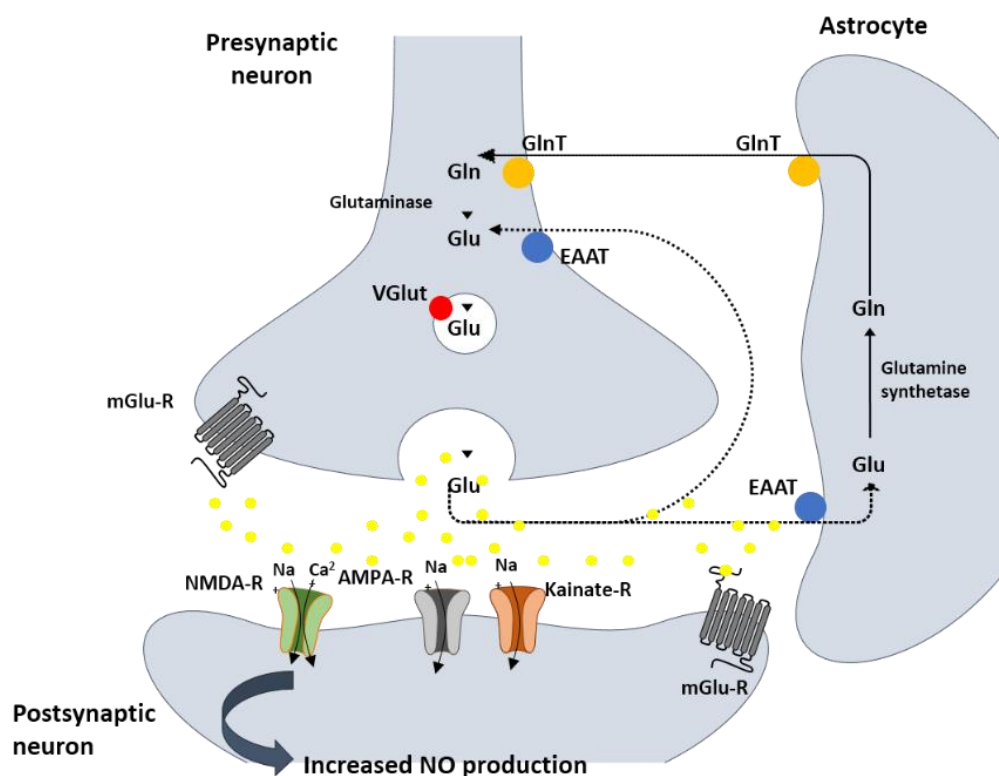


Figure 1.12: Synthesis and regulation of glutamate

Glutamate is regulated via EAATs located on the astrocytic end-feet and converted by the astrocytic enzyme glutamine synthetase (GS) to glutamine, the precursor for the major inhibitory neurotransmitter, GABA, as well as glutamate (Rothman et al., 2012). Glutamine is then released from the astrocyte and can be taken back up by neurons, transformed into glutamate, stored into vesicles and released in the synapse where it can modulate glutamatergic receptors.

There is growing evidence that glutamate is involved in the pathophysiology and treatment of depression. Since astrocytes play a role in the removal of glutamate from the synapse, it has been proposed that an astrocytic deficit may account for alterations in glutamate neurotransmission in depression [for review, (Kugaya and Sanacora, 2005)]. An implication

of reduced astrocyte number is that remaining astrocytes will need to compensate and remove extracellular glutamate at a higher rate in order to avoid an accumulation of extracellular glutamate (Hansson et al., 2000).

7.6. Model of astroglial toxicity

7.6.1. In vitro model of astroglial toxicity

In addition to the neuroprotective/trophic role of astrocytes assessed by CM transfer onto neurons, dysfunctional astrocytes can impair neuronal structure and function. Dysfunctional astrocytes have been shown to contribute to abnormal glutamate concentrations with knock-on disruptions in synaptic plasticity [for review, (Sanacora et al., 2012)]. L- α amino adipic acid (L-AAA) (**Fig 1.13**) is a specific astrocytic toxin acting as a glutamate analogue (Brown and Kretschma, 1998; Nishimura et al., 2000). Once taken up by the astrocytes, L-AAA is involved in competitive inhibition of the GS and γ -glutamylcysteine synthetase, contributing to abnormal glutamate cycling and metabolism. L-AAA causes astrocytic dysfunction. *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 (Tsai et al., 1996; Brown and Kretschma, 1998). L-AAA-mediated astrocyte toxicity may therefore involve glutamate accumulation in astrocytes and/or oxidative stress. Other research demonstrated that 24 h of L-AAA (1mM) exposure on cultured rat astrocytes leads to a reduction in protein synthesis (Nishimura et al., 2000). Amino adipic acid (AAA) occurs naturally in the brain at micromolar concentrations (~30 μ M) and is an intermediate in lysine metabolism (Wu et al., 2015). It is degraded by transamination to α -oxoadipic acid by the enzyme AAA aminotransferase. This enzyme is also responsible for the catalysis of kynurenine to the neuroprotective excitatory amino acid receptor antagonist kynurenic acid (Tsai et al., 1996; Han et al., 2008). 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).

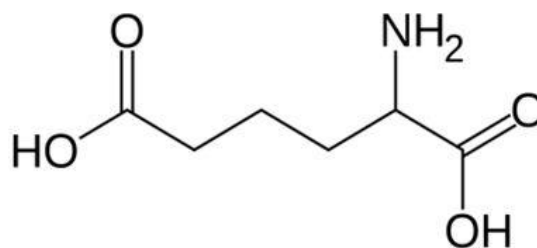


Figure 1.13: Structure of L-AAA

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.

7.6.2. Astrocytic disruption, a new experimental model of depression

It is hard to recreate the complexity of major depression in animals due to the lack of reliable animal models. According to Berton et al. (2012), “continued progress will critically depend on our ability to emulate aspects of depressive symptomatology and treatment response in non-human organisms”.

Because of the relationship between astrocytes, glutamate levels and depression-related behaviour, it appears that astrocytic disruption following the administration of the astroglial toxin L-AAA is a promising animal model to study astrocytic-related glutamate impairment and related depressive behaviours.

A study reported the effect of stereotactically-delivered L-AAA (100µg/µl, 0.5µl, 2 injections) into the PLC of rats on depressive-like behaviours (Banasr and Duman, 2008). The effects of toxin administration were investigated in a variety of behavioural tests, including the sucrose preference test (SPT) and the FST, which are known to be affected by stress and antidepressant treatment. The extent to which this toxin affected the animal’s behaviour is comparable to that induced by the CUS model. Furthermore, this study found that L-AAA administration in the PLC was associated with a reduction in the immunoreactivity of GFAP, specifically in the PLC. GFAP is a cytoskeletal protein principally expressed in the white matter of the brain (Bushong et al., 2002) and is the main intermediate filament in mature astrocytes in the CNS. Although it is believed to only stain 20-30% of the total astrocyte population of a region, comparative analysis of GFAP-positive cells is sufficient to demonstrate decreases in astrocyte cell activation and/or density in post-mortem brains of depressed patients compared to healthy controls (Rajkowska and Stockmeier, 2013). In addition, there are consistent reductions in GFAP immunoreactivity in

astrocytes in the frontal-cortical regions of depressed patients relative to non-psychiatric controls (Miguel-Hidalgo et al., 2000).

Overall, cortical astrocytic disruption (indicated by GFAP immunoreactivity) is associated with a range of depressive-like behaviours in rats. Furthermore, in mice, two consecutive doses of L-AAA into the PLC (100µg/µl, 0.6µl, 2 injections) decreased GFAP immunoreactivity 5 days after the second injection. 4 days after L-AAA delivery, a decrease in glutamate and glutamine concentrations was observed in the PFC (including the PLC), but no changes were observed 2 days after L-AAA administration. Mice with reduced levels of glutamate/glutamine in the PFC also exhibited increased immobility in the FST and lower sucrose preference 4 days after the second L-AAA administration (Lee et al., 2013). In addition, in GFAP null mice, it has been demonstrated that there is an alteration in glial glutamate transporter protein homeostasis, suggesting an impairment in astrocyte physiology that can influence neuronal glutamate transporter trafficking mechanisms (Hughes et al., 2004; Rajkowska and Miguel-Hidalgo, 2007).

In rats, a refinement with a single high dose of L-AAA into the mPFC (20µg/µl, 5µl, 1 injection) resulted in a reduction in the expression of astrocytic cell markers GFAP and S100b 2 and 6 days following L-AAA administration and affected performance in the attentional set-shifting test (AASST) and the working memory in the water-maze. L-AAA also decreased the nuclear neuronal antigen (NeuN), a marker of mature neurons and increased microglia activation at the injection site 2 and 6 days following L-AAA administration. Ionised calcium binding adaptor molecule 1 (Iba1)-positive cells (microglia), exhibited a typical activated morphology, characterised by hypertrophic structure and retracted processes (Lima et al., 2014). In addition, reduced astrocytic function following chronic continuous infusion of L-AAA over 10 days into the left lateral brain ventricle in mice (1µg/µl L-AAA per hour) counteracts the increase of doublecortin (DCX) in the PFC, an immature neuronal marker, following treatment with the hallucinogenic alkaloid harmine. L-AAA blocked the harmine-induced increase in hippocampal neurogenesis (Liu F., et al., 2017).

Astrocytic ablation in the rat brain led to a reduction in apical dendrite length and the number of basal dendrites on pyramidal neurons of the mPFC (Lima et al., 2014), suggesting an effect of glial dysfunction on neuronal complexity. These results indicate that the effect of stress on neuronal complexity as observed in depression could be mediated by astrocytic dysfunction.

Astrocytic dysfunction is sufficient to produce depressive-like behaviours *in vivo* in the absence of stress hormones (Banasr and Duman, 2008; Lima et al., 2014).

In mice, harmine increased BDNF protein levels in the hippocampal and prefrontal areas in CUS-treated mice. This effect was blocked by L-AAA, demonstrating that the astrocytic BDNF may be necessary for the regulation of depressive-like behaviours (Liu F., et al., 2017). This is in line with previous studies showing that astrocytic abnormality induces a decrease in glutamate uptake and cycling, as well as an impairment in BDNF signalling and hippocampal neurogenesis (Martin et al., 2012; Lutgen et al., 2016). Indeed, BDNF has been considered to regulate depressive-like behaviours through the promotion of hippocampal neurogenesis (Jiang et al., 2015a; Kim and Leem, 2016; Sakharkar et al., 2016).

Although the mechanism by which L-AAA exerts its toxicity is still largely unknown, the fact that it affects astrocytes specifically allows for the study of behavioural and physiological changes induced by astrocytic dysfunction. However, because of the lack of astrocytic marker as GFAP is a cytoskeletal marker, it is not really clear whether L-AAA creates astrocytic dysfunction or depletion *in vivo*. Identifying astrocytes is quite limited in the research field because of a lack of specific markers. GFAP is commonly used but is not astrocytic specific and only stain a small percentage of reactive astrocytes.

Nethertheless, L-AAA-induced astrocytic disruption could be a useful animal model to explore the role of astroglial function in depression-related behaviours. Similarly, it would be advantageous as a standalone or complementary model in order to test the efficacy of both existing and novel antidepressant drugs.

8. Aims and objectives

This investigation firstly aims to characterise the impact of astrocytic dysfunction on neurons *in vitro*, specifically the effects of L-AAA-induced astrocytic dysfunction on neurite outgrowth and synapse number in primary cortical neurons from rats. In addition, to further characterise the mode of L-AAA-induced astrocytic toxicity, the effect of L-AAA on astrocytic oxygen consumption rates and glycolysis is investigated.

The second part of this project is focused on the translation from *in vitro* experiments to an animal model of L-AAA-induced astrocytic dysfunction. The L-AAA brain delivery model is refined by carrying out dose- and time-dependent experiments in C57Bl6/J mice on the effects of intra-cortical administration of L-AAA. Behaviour of the mice was assessed.

Furthermore, the effect of L-AAA on neuronal complexity, dendritic spine density and microglial and astrocytic reactivity is investigated in post-mortem brain from these mice.

Finally the effect of targeting the neuronal NMDA-R/NO signalling pathway is assessed as a strategy to promote antidepressant activity and prevent neuronal atrophic effects following L-AAA-induced astrocytic impairment.

Chapter 2

Material and Methods

I. Materials

1. General consumables and equipment

1.1. Consumables

6-, 24- and 96-well plates	Sarstedt, Ireland
Calibrated pipets (microcapillary 1-5µl)	Sigma-Aldrich
Cell culture flasks (T-75cm ²)	Corning, Ireland
Cell scrapers	Sarstedt, Ireland
Cell strainers (40µm),	BD Falcon VWR, Ireland
Containers (70ml)	Sarstedt
Falcon tubes (15ml, 50ml),	Sterile Sarstedt, Ireland
Filter units (0.22µm), single-use (Millex)	Millipore, Ireland
Glass coverslips (13mm, 35mm)	VWR, Ireland
Glass coverslips 22mm x 50mm	Fisher Scientific
Haemocytometer	VWR, Ireland
Microscope slides 76mm x 26mm	Fisher Scientific, Ireland
Microtome blades (c35 type)	Lab. Instr. & Supply
Microtubes (0.5ml, 1.5ml, 2ml)	Sarstedt, Ireland
Parafilm	Fisher Scientific, Ireland
Pasteur pipettes (3.5ml)	Sarstedt, Ireland
PCR tubes (0.2ml)	Sarstedt
Pipette tips (10µl, 200µl, 1000µl)	Sarstedt, Ireland
Pipettes (5ml, 10ml, 25ml), sterile	Sarstedt, Ireland
Petridishes (90mm)	Fisher Scientific, Ireland
Scalpels, disposable, sterile (Swann-Morton)	Fisher Scientific, Ireland
Syringes (20ml, 50ml)	Fisher Scientific, Ireland
XF24-well cell culture plate	Seahorse, Biosciences

1.2. General equipment

Autoclave	Sanyo, Ireland
Axio Imager Z1	Carl Zeiss
Axiovert 200M	Zeiss
Biosafety cabinet	ADS Laminaire, France
20x IX81-long focal length Fluorescent microscope	Olympus

Centrifuge: Legend RT+	Sorvall, Ireland
Cryostat	Leica
Vibratome	Leica
Gyro rocker	Stuart
Haemocytometer	VWR, Ireland
Incubator (Innova CO-48)	New Brunswick, Ireland
Micropipette puller (P-87)	Sutter Instrument
Microscopes CKX41	Olympus
Nanodrop® ND1000 Spectrophotometer	Thermo Fisher Scientific
Needles, hypodermic (25G)	Microlance BD
Peristaltic pump	Gilson
Plate reader	Biotek
PH meter	Mettler-Toledo
Vortex	Ika, Ireland
Scale	Denver Instrument, Ireland
Scale (precision)	Mettler-Toledo, Ireland
SP8 confocal microscope	Leica
Syringes, plastic (1ml)	B-Braun
Water bath	Grant, Ireland
XF24 Analyzer	Seahorse, Biosciences

2. In vitro studies

2.1. Animals

1-day-old Wistar rats (male/female)	Comparative Medicine, TCD
2/3-day-old Wistar rats (male/female)	Comparative Medicine, TCD

2.2. Biochemical assays

2-deoxy-D-glucose	Sigma Aldrich
Absolute ethanol	Hazmat, TCD
B-27 supplement	Invitrogen, Ireland
Pierce BCA Protein Assay Kit	Fisher
Biocidal ZF™	WAK-Chemie Medical, Germany
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazine	Sigma-Aldrich, Ireland
Dulbecco's modified Eagle medium (DMEM)	Sigma-Aldrich, Ireland

Dulbecco's phosphate buffered saline (DPBS) (1x)	Invitrogen, Ireland
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Ireland
DPBS (10x)	Invitrogen, Ireland
Fetal bovine serum (FBS) heat inactivated	Biosera, UK
Fungizone	Invitrogen, Ireland
Glucose	Sigma-Aldrich, Ireland
Glutamax	Invitrogen, Ireland
GFP DNA plasmid	University of Turku
Lipofectamine 2000	Invitrogen
Methanol	Fisher Scientific, Ireland
Neurobasal A medium	Invitrogen, Ireland
Normal goat serum	Sigma-Aldrich, Ireland
Oligomycin	Calbiochem
Paraformaldehyde	Sigma Aldrich
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-Ethylenediaminetetraacetic acid (EDTA) solution (0.25%)	Sigma-Aldrich, Ireland
Un-buffered DMEM	Seahorse, Biosciences
Vectashield mounting medium with DAPI	Vector Laboratories, UK
Virkon	Antec International, USA
XF Calibrant	Seahorse, Biosciences

2.3. Experimental treatments

Ketamine (hydrochloride) (Vetalar V®)	Pfizer, Ireland 46
L-2-Aminoadipic Acid (L-AAA)	Sigma-Aldrich, Ireland
2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl)-4,6-dichlorophenol (IC87201)	
4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxy	TopChem, Ireland

benzoic acid (ZL006)	TopChem, Ireland
1-[2-(trifluoromethyl)phenyl] imidazole (TRIM)	Alfa Aesar, UK

2.4. 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

2.5. Primary and secondary antibodies for immunocytochemistry

2.5.1. Primary antibodies

Table 2.1: Primary antibodies used for immunocytochemistry

Name	Host species	Company	Catalogue number	IHC dilution
B-III-tubullin	Mouse	Promega, Ireland	G7121	1:1000
Synaptophysin	Rabbit	Invitrogen	PA1-1043	1:500
PSD-95	Mouse	Invitrogen	11508772	1:500

2.5.2. Secondary antibodies

Table 2.2: Secondary antibodies used for immunocytochemistry

Name	Host species	Company	Catalogue number	IHC dilution
Alexa 488	Goat (Anti mouse)	Invitrogen Ireland	A11029	1:1000 1:2000
Alexa 546	Goat (anti rabbit)	Life technologies	A11010	1:1000

3. In vivo studies

3.1. Animals

C57Bl6/J mice (male), 9-14 weeks old

Charles River or Harlan, UK

Tg(Thy1-cre/ERT2,-EYFP)HGfng (male), 12weeks old

Jackson laboratory,
BioResources, Trinity College

3.2. Chemicals and reagents

3,3'-diaminobenzidine (DAB)

Dako Laboratories

Bovine Serum Albumin 96% (BSA)

Sigma Aldrich

Corn oil

Mazola

Ethanol (ETOH)

Sigma Aldrich

Elite Vectastain[®] ABC kit

Vector laboratories, UK

DPX mounting medium

Fisher Scientific, Ireland

Heparin Sodium

Wockhardt

Hydrochloric Acid (HCl)

BDH Chemicals

Hydrogen peroxide solution, 30% w/w in H₂O (H₂O₂)

Sigma-Aldrich, Ireland

Gelatin

Sigma-Aldrich, Ireland

Isopentane

Sigma Aldrich

Methanol

Sigma Aldrich

Normal Goat Serum (NGS)

Sigma Aldrich

Paraformaldehyde

Sigma Aldrich

Saccharin

Sigma-Aldrich

Sodium Chloride (NaCl)

BDH Chemicals

Sodium Dihydrogen Phosphate (NaH₂PO₄)

Sigma Aldrich

Sodium Hydrogen Phosphate (Na₂HPO₄)

Sigma Aldrich

Sodium Hydroxide (NaOH)

Sigma Aldrich

Sucrose

Sigma Aldrich

Tamoxifen

Sigma

Tissue-tek OCT

compound Sakura

Triton-X

Sigma Aldrich

Xylene

Sigma-Aldrich

3.3. Equipment and apparatus for surgical procedures and behavioural testing

Behavioural recording system (Ethovision 3.1)	Noldus, the Netherlands
Open field circular arena (72cm)	Custom made
Pyrex beakers (2l)	Fisher Scientific, Ireland
Mice ear bars	Kopf Instruments, Ireland
Stereotactic frame	Kopf instruments, Ireland
Tail suspension test apparatus	Custom made

3.4. Animal care

Betadine	
EMLA 5% w/w cream	AstraZeneca
Euthanimal (Pentobarbital)	Alfasan
Ethylcalm	Invicta
EMLA (lidocaine, prilocaine)	AstraZeneca
Lubrithal	Dechra
Tissue adhesive (Surgibond®)	SMI AG

3.5. Experimental treatments

Evans blue	Sigma-Aldrich Ireland
Ketamine (hydrochloride) (Vetalar V®)	Pfizer, Ireland 46
L-2-Aminoadipic Acid (L-AAA)	Sigma-Aldrich, Ireland
1-[2-(trifluoromethyl)phenyl] imidazole (TRIM)	Alfa Aesar, UK
4-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxy benzoic acid (ZL006)	TopChem, Ireland

3.6. Primary and secondary antibodies for immunohistochemistry

3.6.1. Primary antibodies

Table 2.3: Primary antibodies used for immunohistochemistry

Name	Host species	Company	Catalog number	IHC dilution
GFAP	Rabbit	Dako	Z0334	1:1000
IBA1	Rabbit	Wako	019-19741	1:3000
NeuN	Mice	Millipore	MAB377	1:300

DCX	Mice	Santa cruz	SC-271390	1:250
S100b	Rabbit	Abcam	Ab868	1:1000

3.6.2. Secondary antibodies

Table 2.4: Secondary antibodies used for immunohistochemistry

Name	Host species	Company	Catalog number	IHC dilution
Alexa 488	Goat (anti rabbit)	Invitrogen, Ireland	A11029	1:1000
Alexa 488	Goat (anti mouse)	Invitrogen, Ireland	A-11001	1:500 1:1000
Alexa 546	Goat (anti rabbit)	Life technologies	A11010	1:1000

I. Methods

1. In vitro experiments

1.1. Preparation

1.1.1. Aseptic techniques

Aseptic techniques were used in order to prevent any fungal, bacterial and viral contamination and to maintain a sterile environment and healthy cells. All the experiments were performed under the laminar flow hood, allowing only filtered air to come into contact with cells, thus preventing contamination with airborne pathogens. The surfaces of the fume hood were swiped with 70% ethanol and pipette tips, Eppendorf tubes and distilled water DH₂O were sterilised by autoclave at 120°C for 1h prior to use. The equipment (dissection kit) was cleaned with Virkon and all the falcon tubes and plates were plastic-wrapped and sterile. The hood was exposed to UV rays for 30 min for sterilisation to avoid any pathogen contamination. Both the fume hood and the incubator were cleaned with Biocidal to maintain a sterile environment. A lab coat and disposable plastic gloves were worn during the entire duration of the experiments and sprayed with 70% EtOH before use.

1.1.2. Preparation of culture media

1.1.2.1. *Dulbecco's modified Eagle's medium*

Dulbecco's modified Eagle's medium (DMEM) Ham's F12 (500ml) was used for primary glial cells and was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (50ml), 1% penicillin-streptomycin (5ml) and 0.1% fungizone (500µl). All the supplements were filter-sterilised using a 0.2µm syringe filter and added to a bottle of DMEM. The DMEM was stored in the fridge at 4°C for further use.

1.1.2.2. *Neurobasal A medium*

Neurobasal A medium (NBM) (500ml) was used for primary neuronal cultures and was supplemented with 1% glutamax (5ml), 1% penicillin-streptomycin (5ml), 0.1% fungizone (500µl). All the supplements were filter-sterilised using a 0.2 µm syringe filter and added to a bottle of NBM. The NBM was stored in the fridge at 4°C for further use. When required, a working solution was prepared by adding 1% of B-27 to NBM (500µl for 50ml).

1.1.2.3. *Phosphate buffered saline*

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

1.1.3. Drugs preparation

All stock solutions were filter-sterilised using a 0.2µm syringe filter and frozen at -20°C in aliquots for future use. Stock solutions were diluted to working concentrations in appropriate pre-warmed media prior to use (DMEM or DMSO).

Table 2.5: Preparation of stock solutions

Compound name	Molecular weight (g/mol)	Stock Concentration	Dissolved in:
IC87201	309	20 mM	DMSO
Ketamine	238	20 mM	NBM
L-AAA	161	25 mM	NBM
ZL006	328	1 mM	NBM
TRIM	212	10mM	NBM

1.2. Preparation and culture of primary glial cells

1.2.1. Collection of primary cortical mixed glial cells

Cortical brain tissue was collected from post-natal day 2 or 3 (P 2-3) Wistar rat pups from TCIN's breeding colony in the cell culture room under the laminar flow hood with sterile conditions. Pups were decapitated using large scissors, smaller scissors were used to cut away the skin along the midline (from the back of the head to the nose) to expose the brain, firstly through the skin and then through the skull. A straight forceps was used to peel off the skull and a curved forceps was used to remove the brain.

The brain was then placed on a Petri Dish with a drop of pre-warmed DMEM to avoid dryness. A straight forceps was used to scrape away meninges and blood vessels. Cortical tissue from both hemispheres was subsequently dissociated from the rest of the brain. The cortices from the rat pup were then cross-chopped using a sterile scalpel and remaining blood vessels were removed. The chopped cortical tissue was placed in 6ml of pre-warmed DMEM and left in the incubator for 20 min at 37°C. After, the cell suspension was triturated until homogenous with a pipette and passed through a sterile cell strainer (40µm) into a 50ml falcon tube in order to remove any blood vessels. The falcon tube was wrapped with parafilm and then centrifuged at 2000 rpm for 3 min at 20°C. The supernatant was removed and discarded and the pellet was re-suspended in 1ml of pre-warmed DMEM until obtaining a homogenous cellular suspension. 4ml of pre-warmed DMEM were added into an empty flask and spread across the bottom, 1ml of cell suspension was then placed into the flask to obtain a total volume of 5ml. The flasks were placed for 2 h in an incubator at 37°C with 5% CO₂ to allow cell adherence. After 2 h of incubation, the flasks were flooded with 5 additional ml of DMEM to obtain a total volume of 10ml per flask. The day of plating was referred to as day in vitro 0 (DIV 0). Media was replaced every 4-5 days by removing the old media and gently adding 10ml of fresh, pre-warmed DMEM to the sides of the flask in order to prevent disturbing the cells. Glial cells were confluent and were separated at approximately DIV 8-12.

1.2.2. Astrocyte isolation and re-plating

To obtain purified isolated cultures of astrocytes, primary mixed glial cells were cultured from post-natal day 2 or 3 (P 2-3) Wistar rat pups as described above. At approximately DIV8-12, when glial cells started to become confluent, the flasks were wrapped with parafilm and agitated on an orbital shaker for 2h at 200 rpm to separate the non-adherent microglia and

oligodendrocyte cells from the astrocytic cells fixed to the flask. The flasks were then struck firmly 10 times in the laminar flow hood for the microglia and oligodendrocytes to fully detach from the surface of the flasks. The media containing microglia and oligodendrocytes was then removed and discarded. 3ml of pre-warmed DMEM was added to the remaining astrocytic monolayer and astrocytes were scraped from the bottom of the flask, rinsed with an additional 2ml of DMEM and then collected and placed into a falcon tube. The falcon tube was protected with parafilm and was centrifuged at 2000 rpm for 3 min at 20°C. The supernatant was discarded and the pellet re-suspended in 1ml pre-warmed DMEM.

For CM experiments, the cells were diluted into 6ml of DMEM and 100µl was put into each well of two 24-well plates. 100µl of the solution was placed into each well and cells were allowed to seed on the plate for 2h. After that, an additional 200µl of pre-warmed DMEM were added into each well to obtain a total volume of 300µl.

With regard to the seahorse experiment, astrocytes were diluted with DMEM at a dilution of 1:5 (900µl of the supernatant was mixed with 3.6ml of DMEM to obtain a dilution of 1:5). 150µl of the solution was placed into each well and cells were allowed to seed on the plate for 4h. After that, an additional 150µl of pre-warmed DMEM were added into each well to obtain a total volume of 300µl.

1.2.3. Astrocyte treatments for CM

To obtain astrocyte CM, cells were grown in 24-well plates and treated with a volume of 300µl of compounds (L-AAA or NBM) diluted in pre-warmed NBM for 24 h. After this time, the resulting CM from each well of a particular treatment was collected with a Pasteur pipette and pooled together in a 15ml falcon tube before being aliquoted. The resulting supernatant was collected and filter-sterilised using a 0.2µm syringe filter. B-27 at a dilution 1:100 was added to the CM before storage at -20°C for further use (**Fig 2.1**).

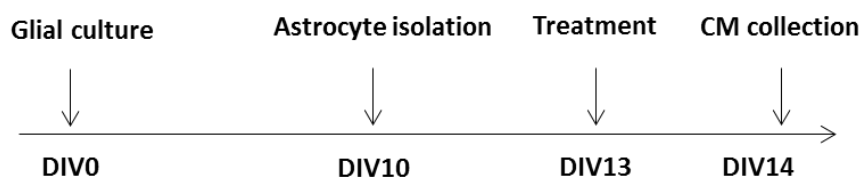


Figure 2.1: CM treatment and collection

Glial cells were prepared from 2/3 day old Wistar rat pups and allowed to grow until around DIV10. When the cells were confluent, the astrocytes were isolated and placed into 24-well plates as previously described. After 3 days the astrocytes were treated with either control solution (DMEM) or L-AAA and the resultant CM was collected and filter-sterilised on the following day.

1.3. Preparation and culture of primary neurons

1.3.1. Preparation of coverslips

Poly-D-lysine was prepared by reconstituting 5mg with 50ml of sterile H₂O to obtain a stock concentration of 0.1mg/ml. This solution was then filter-sterilised using a 0.2µm syringe filter and frozen at -20°C in 5ml aliquots until further use. When needed, the Poly-D-lysine aliquot was diluted by the addition of 5ml sterile dH₂O giving a working concentration of 50 µg/ml. Glass coverslips were placed into 24-well plates and further sterilised by exposing them under UV light for 1 h. Poly-D-lysine (75µl) was subsequently added into the centre of each coverslip and incubated for 45 min at 37°C. Following this, the poly-D-lysine solution was removed and kept at 4°C to be re-used. The coverslips were then rinsed twice with sterile dH₂O. The plates were left to fully dry for at least 2 h until further use and small drops of water were removed with a Pasteur pipette. Plates were then stocked for up to one week in the fridge or frozen at -80°C.

1.3.2. Preparation of primary cortical neuronal cultures

Neuronal cultures were prepared from post-natal day 1 (P1) Wistar rat pups as described previously (McNamee et al., 2010; Day et al., 2014). Trypsin-EDTA, B-27, NBM, DMEM and plates were pre-warmed in the incubator before starting the dissection. The cortex was dissected using the same technique as for astrocyte cell preparations and the tissue was finely cross-chopped with a scalpel in a drop of pre-warmed DMEM. The tissue was then transferred into trypsin-EDTA (5ml), and incubated for 2 min at 37°C. DMEM (5ml) was added to the trypsin as FBS helps to deactivate the trypsin. The tissue was gently 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 DMEM (5ml). This cell suspension was triturated and passed through a sterile cell strainer (40µm) and centrifuged at 2,000 rpm for 3 min at 20°C. The supernatant was discarded and the pellet was re-suspended in 1ml of pre-warmed NBM + B-27 (dilution 1:100) and gently triturated until a homogenous cellular suspension was obtained.

1.3.3. Neuronal cell counting and plating

The neuronal cell suspension in the 1ml was counted using the trypan blue exclusion method. Cells were first diluted at a dilution of 1:50 in NBM and again at 1:2 with trypan blue leading

to a total dilution of 1:100. The cells were 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 100 μ l of this dilution was gently pipetted on the centre of a poly-D-lysine coated coverslip in 24-well plates. The plates were left in an incubator at 37°C with 5% CO₂ for 2 h to allow adherence of the cells to the coverslip, followed by the addition of 200 μ l of NBM. 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 for 20 days by replacing the media with fresh pre-warmed NBM every 3-5 days. These neurons were treated with compounds at around DIV 19/20 and fixed for subsequent immunocytochemistry and imaging.

1.4. RNA Analysis

1.4.1. 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 μ l RA1 lysis buffer (supplied in Total RNA isolation kit; 350 μ l) containing 1% β -mercaptoethanol was pipetted into each of the wells. Cells were scraped from the base of the wells using a 1000 μ l filtered pipette tip and the lysate was transferred to a 2ml 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.

1.4.2. 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 μ l 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 (2ml) and 350 μ l 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 1.5ml microcentrifuge, which contained 10 μ l of reconstituted rDNase to 90 μ l rDNase reaction buffer. 95 μ l 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 deoxyribonucleic acid (DNA) on the membrane. Firstly, 200µl 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 600µl RA3 buffer (to which 50ml of 200-proof ethanol had been added to the 25ml RA3 buffer concentrate) and centrifuged at 11000 x g for 30 s. The third and final wash entailed adding 250µl 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 60µl 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.

1.4.3. 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 A260/280 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.

1.4.4. 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.

1.4.5. Real-time PCR (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 μ l volume of the diluted cDNA samples were pipetted into a PCR plate, along with 0.5 μ l of target primer, 0.5 μ l of β -actin primer, and 5 μ l of Taqman® Universal PCR Master Mix. Electronic pipettes (EDP3 20-200 μ l, 2-20 μ l, and 10-100 μ l) were used in order to ensure accuracy. RT-PCR was carried out using Step One 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 expressions 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 2.6: 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
S100b	Rn00566139_m1

1.5. 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 bio-reduction of the dye reduces the amount of the oxidized form (blue) and concomitantly increases the fluorescent intermediate (red). A 440 μ M solution of Alamar Blue dye was prepared by dissolving 25mg resazurin in 226ml dH_2O . This stock solution was then stored in a dark bottle for future use. A working concentration of 44 μ M resazurin was prepared by diluting resazurin 1:10 in the appropriate pre-warmed fresh NBM for neuronal assays and DMEM 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 μ l was added per well (30 μ l Alamar Blue, 270 μ l media). Plates were incubated for 2-4 h at 37°C in a humidified, 5% CO_2 atmosphere. Following a colour change, aliquots of supernatants (100 μ l) were transferred in triplicate to a 96-well flat-bottomed plate. Absorbance was read at 600nm 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

1.6. Immunocytochemistry

1.6.1. Fluorescent immunocytochemistry for Sholl analysis

Fluorescent immunocytochemistry was performed on DIV-4 neurons grown on glass coverslips with Poly-D-lysine. Cells were washed under sterile conditions in the laminar flow hood once with 1 x PBS, fixed with ice-cold methanol (300 μ l per well) for 20 min at -20°C and washed three times with 1 x PBS. All the following steps were performed outside the sterile fume hood on the laboratory bench. 4% NGS diluted in 1 x PBS for 2 h at room temperature was used to block the non-reactive sites (300 μ l per well). The blocking buffer was then removed and 300 μ l of mouse BIII-Tubulin primary antibody (1:1000, Promega Ireland) was diluted in 1 x PBS. The cells were incubated in primary antibody overnight at 4°C. The next day, cells were washed three times in 1ml 1 x PBS. Cells were incubated with

the secondary antibody goat anti-mouse Alexa 488 (1:2000 into 1 x PBS, 200µl, Invitrogen). The plates were protected from light to avoid any loss of fluorescence for 2h at room temperature. Lastly, the cells were washed three times in 1ml 1 x PBS. The glass coverslips were then carefully removed with a forceps and a needle and mounted onto glass slides with Vectashield fluorescent DAPI (4',6-diamidino-2-phenylindole) mounting media. Each coverslip was sealed with nail varnish and stored in the dark at 4°C until visualised with a fluorescence microscope.

1.6.2. Fluorescent immunocytochemistry for the quantification of co-localised puncta

Neurons around DIV 20 were treated for 24 h and subsequently fixed in methanol as described for the Sholl analysis. 50% NGS diluted in 1 x PBS with 0.2% Triton-X-100 was used to block the non-reactive sites (300µl per well) for 2h at room temperature. The blocking buffer was then removed, cells were rinsed in 1ml 1 x PBS and 300µl of primary antibody cocktail (Synaptophysin; rabbit, thermos scientific, 1:500 and post-synaptic density-95 (PSD-95); mouse, fisher scientific 1:500) was added. The cells were incubated with a primary antibody cocktail overnight at 4°C. On day 2, the cells were washed once in 1 x PBS, stained with the secondary antibodies (Alexa 546 goat anti rabbit and Alexa goat anti mouse 488) diluted at 1:1000 into 1 x PBS (200µl) and kept away from light for 2h at room temperature. The glass coverslips were then removed and mounted onto glass slides with Vectashield fluorescent mounting media containing DAPI. Each coverslip was sealed with nail varnish and stored in the dark at 4°C until visualised with a fluorescence microscope.

1.7. Image analysis

1.7.1. Sholl analysis

The Sholl analysis procedure was adapted from Sholl (1953) and Gutierrez and Davies, (2007). β -III tubulin-stained neuronal coverslips were visualised with the epifluorescent Axio Imager Z1 microscope at x200 magnification (20x objective). This microscope was equipped with a CCD camera and Axiovision software allowing direct visualisation on the screen. Isolated neurons per coverslips were selected for the analysis to avoid any confusion during the analysis. Around 5 individual neurons from each coverslip and around 6 coverslips per experimental condition were imaged and analysed for Sholl analysis with 3 independent biological replicates. Experimental groups were blinded. Neuronal morphology was assessed by looking at the number of primary neurites, number of neuritic branches, neuritic length and the complete Sholl profile.

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 $10\mu\text{m}$ 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 was clearly divided in two parts by at least $5\mu\text{m}$. 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 micrometres 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 for each concentric ring (**Fig 2.2**). Data was collected with the use of a simple computer program (Matlab R2012b, MathWorks, Cambridge, UK.) script.

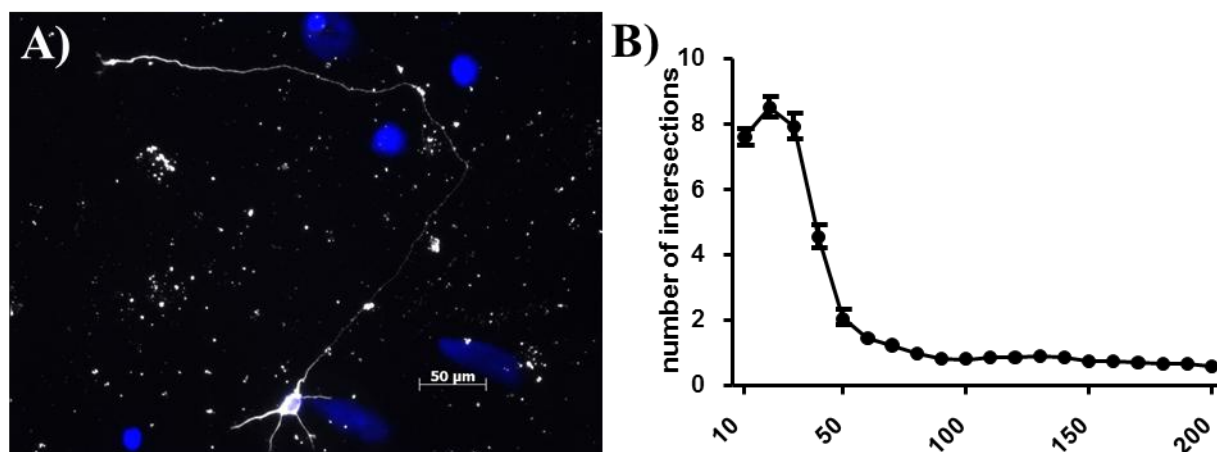


Figure 2.2: Sholl analysis

Representative image of a non-treated rat cortical neuron stained for β -III tubulin and DAPI (A). A typical Sholl profile from non-treated primary cortical neurons at DIV 5 (B). Scale bar = 50 μ m, n = 52 neurons.

1.7.2. Synaptogenesis analysis for the quantification of co-localised puncta (PSD-95/Synaptophysin)

Neurons around DIV 20 were treated for 24 h with different types of CM. This procedure was adapted from (Ippolito and Eroglu, 2010).

Synaptophysin and PSD-95 stained neuronal coverslips were visualised at x400 magnification (40x objective) on an Axio Imager Z1 epifluorescent microscope equipped with a CCD camera and Axiovision software. Around 5 individual images were captured from each coverslip and 6 coverslips per experimental condition were analysed with 3 replicates from independent experiments. Groups were blinded. Using the ImageJ analysis program (National Institutes of Health) with the Puncta Analyzer plug-in, the number of co-localisations was quantified. For image analysis, individual images were opened in ImageJ and the region of interest was manually selected around the neuron. The threshold was then determined for both the red channel (synaptophysin expression) and green channel (PSD-95 expression) in order to visualise discrete individual puncta (**Fig 2.3**). Minimum puncta size was set at a value of 4 for each experiment. The threshold value was standardised based on a value established for a control-treated neuron in order to maintain a consistent approach for comparison between groups.

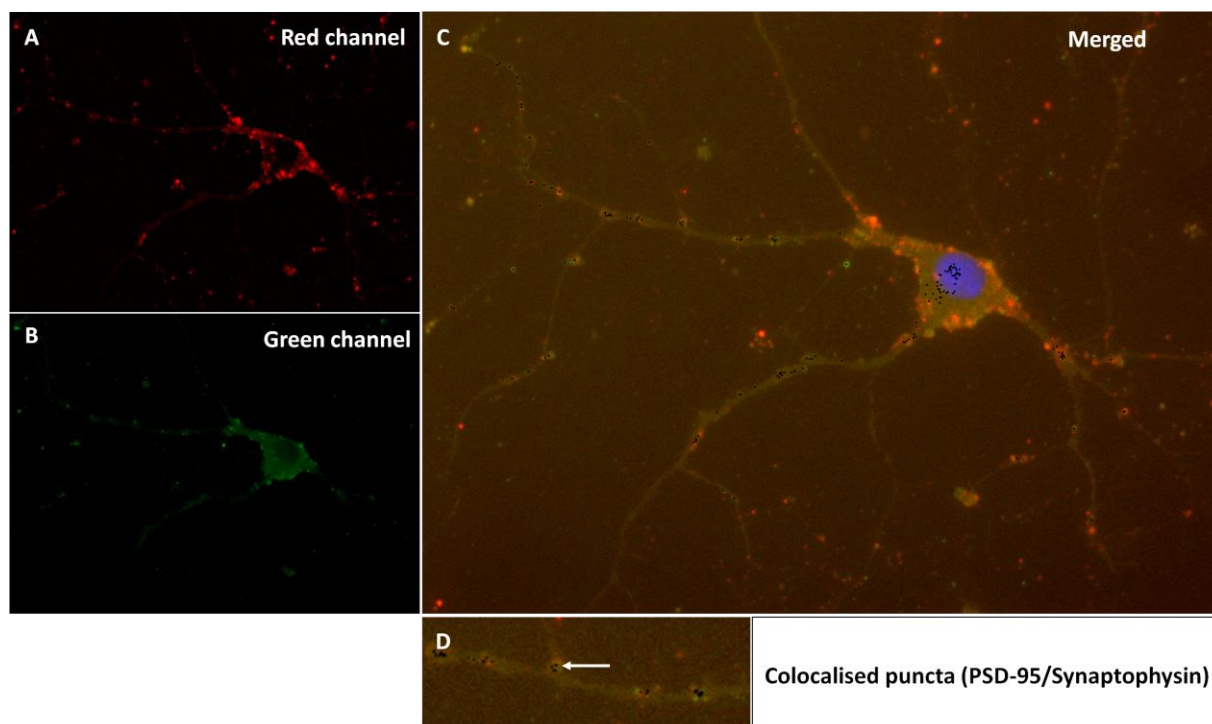


Figure 2.3: Colocalisation of synaptophysin and PSD-95

Primary cortical neurons were treated at around DIV 20 for 24 h before fixation. **(A)** Synaptophysin staining (red channel) **(B)**; Post-synaptic marker, PSD-95 (green channel); **(C)** Merged image of synaptophysin, PSD-95 and the DAPI nuclear stain (blue); **(D)** Individual synaptic co-localised puncta (black dots). Quantification of synapse number was conducted using the ImageJ software.

1.8. GFP transfection

Hippocampal neurons were transfected at DIV7. First, 1 μ l of Lipofectamine 2000 (LF2000) was diluted with 49 μ l of NBM and incubated for 10 min at 37°C. To obtain 30% of transfection efficacy, 150ng of DNA was diluted into 50 μ l of NBM (100% transfection is obtained with 500ng per μ l). After incubation, DNA-mix medium and LF2000-medium were mixed and incubated for 30 min at room temperature.

NBM was removed from the wells and stored in a closed sterile tube in the incubator and replaced with 400 μ l per well of NBM (without antibiotics). 100 μ l of DNA/LF2000 mixture was added to each well and incubated for 40min at 37°C in the incubator and the normal media was put back again.

Cells were treated for 24 h with L-AAA CM or control CM at DIV 15. At DIV16, 300 μ l of paraformaldehyde (PFA) was topped up into each well and cells were incubated for 30 min. Wells were washed 3 times with PBS for 5 min. The glass coverslips were then removed and mounted onto glass slides with Vectashield fluorescent mounting media containing DAPI. Each coverslip was sealed with nail varnish and stored in the dark at 4°C until visualised with a fluorescence microscope (**Fig 2.4**).

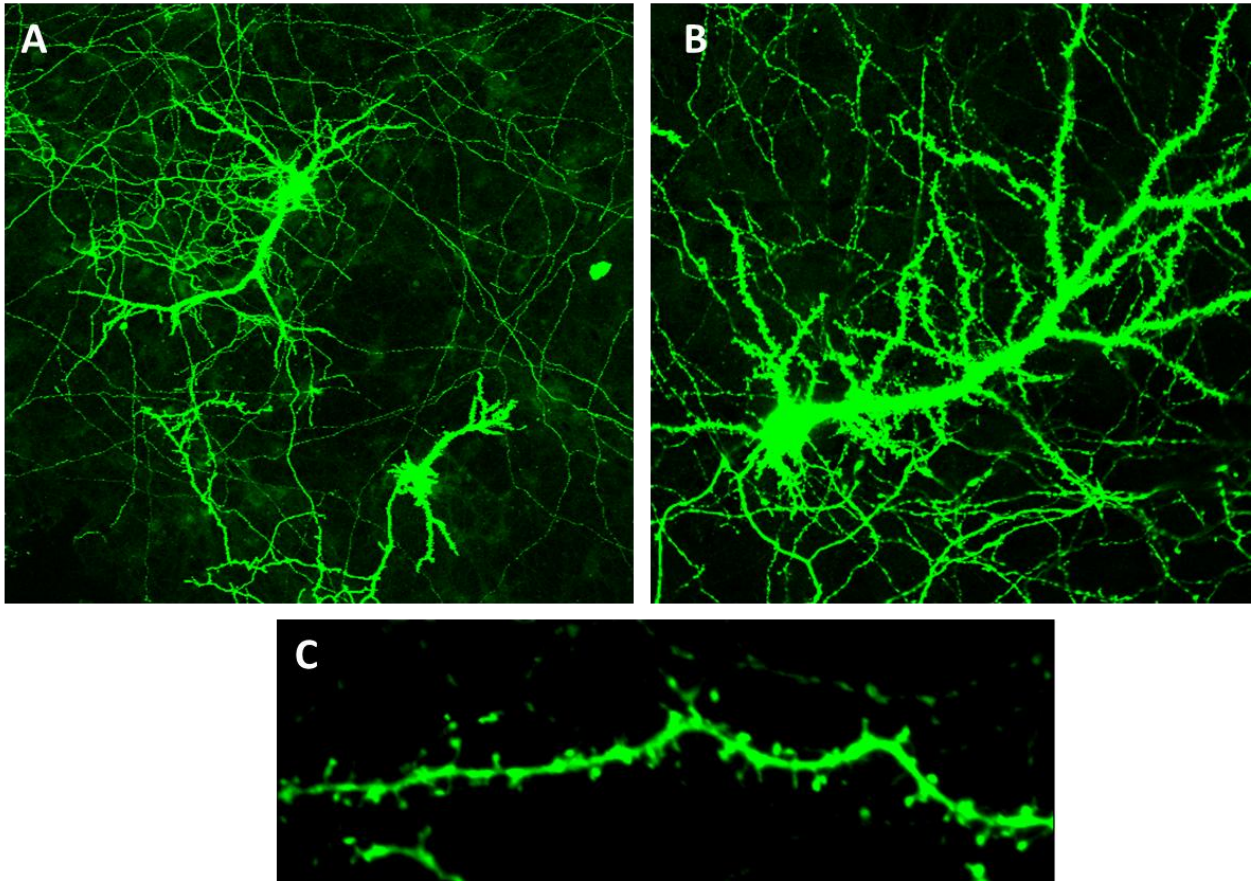


Figure 2.4: GFP transfected neurons

Primary hippocampal neurons were transfected at around DIV 7 with LF2000 and GFP DNA. At DIV15, neurons were treated for 24 h with L-AAA-CM or control CM and were subsequently fixed and imaged with a confocal microscope to perform spine analysis. A) and B) general view of a neuron and C) spines on a dendritic segment.

1.9. Glycolysis and mitochondrial respiration measures in astrocytes

1.9.1. Astrocyte preparation and treatment

For the sea-horse experiment, purified astrocytes were treated at approximately DIV 13 with L-AAA diluted in pre-warmed DMEM at a concentration of 0.5mM for 24 h in a total volume of 500µl of treatment per well into XF24-well plates for 24 h (**Fig 2.5.**)

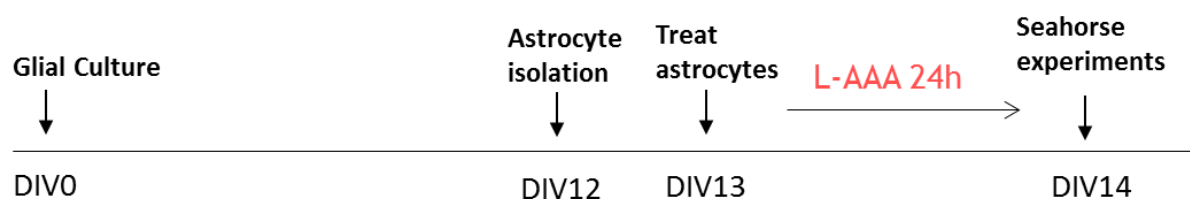


Figure 2.5: Astrocytic culture, isolation and treatment:

Cortical brain tissue was collected from postnatal day 2-3 (P 2-3) Wistar rat pup. At around DIV 12, when mixed glia became confluent, astrocytes were isolated and re-plated into the XF24 sea-horse plate. Astrocytes were allowed to adhere and at DIV 13, astrocytes were treated for 24 h with L-AAA (0.5mM) or a control treatment (DMEM). At DIV14, seahorse measurements were obtained.

1.9.2. Mitochondrial respiration and glycolysis measurement

1.9.2.1. Seahorse plate preparation

On the day prior to oxygen consumption rates (OCR) analysis, the sensor cartridge of the Seahorse apparatus was hydrated by adding Seahorse XF Calibrant solution (1ml) into each well of the utility plate and left overnight in a non-CO₂ incubator at 37°C.

On the day of analysis, after removing the treatment solution (L-AAA 0.5mM) or DMEM for control wells and blanks, 500µl of unbuffered sea-horse media supplemented with 10mM glucose was added slowly to each well, removed, and 600µl of the same unbuffered media supplemented with glucose was added and left for 1 h at 37°C in a 21% O₂ incubator before transfer to the XF24 analyser.

The calibration plate was placed for 5 min into the 37°C, 21% O₂ incubator and then for 25 min in the sea-horse apparatus to calibrate the plate. The inhibitors were diluted into unbuffered sea-horse media and put into wells A, B, C and D of the sensor as follow: Oligomycin (2µM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.5µM), Rotenone A (0.1µM)/Antimycin A (4µM) and 2-Deoxy-D glucose (2-DG) (30mM) were injected at a volume of 75µl in each well of the seahorse plate (**Fig 2.6**)

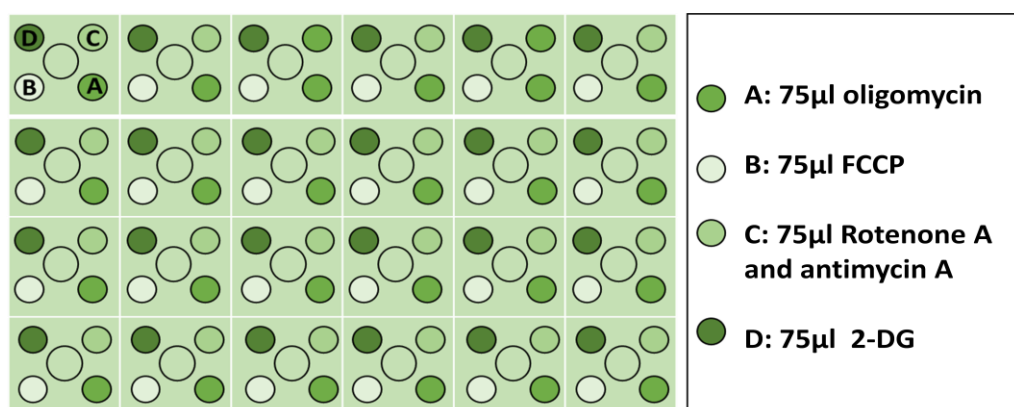


Figure 2.6: Plate treatment

1.9.2.2. Measurement of OCR using Extracellular Flux Analyser

The experiment consisted of 4 basal measurements followed by the injection of an inhibitor, 2 min mixing, 2 min wait, and 2 min measurement cycle (with 2 measures after injection of each inhibitor). Lastly, after the injection of the fourth inhibitor, 4 measurements closed the experiment. 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 cultured plate (Ferrick et al., 2008). The experiments were performed at 37°C. OCR is an indicator of mitochondrial respiration that gives insights into basal respiration, ATP-linked respiration, proton leak respiration, maximal respiratory capacity and non-mitochondrial respiration. Real-time measurements of OCR are made by isolating an extremely small volume (about 2µl) of medium above a monolayer of cells within a microplate. Cellular oxygen consumption (respiration) causes rapid and easily measurable changes to the concentrations of dissolved oxygen in this "transient microchamber" (**Fig 2.7**).

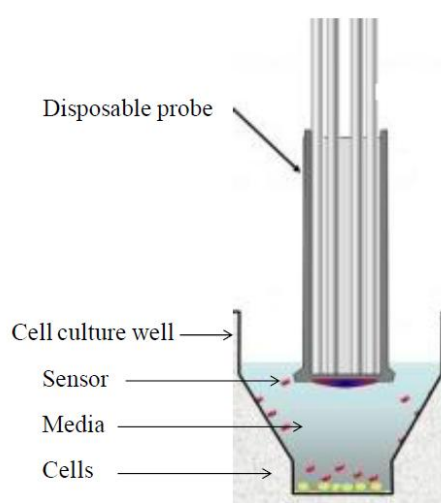


Figure 2.7: Layout of the XF24-well cell culture plates, Seahorse apparatus (Biosciences)

A cutaway view of a well of the XF24 cell culture plate and sensor is shown above. Image adapted from Seahorse (Biosciences).

Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitor oligomycin (2µM), a complex V inhibitor which inhibits ATP synthase. The resulting OCR is used to derive ATP-linked respiration (by subtracting the oligomycin rate from baseline cellular OCR) and proton leak respiration (by subtracting non-

mitochondrial respiration from the oligomycin rate). In this experiment, we used FCCP (0.5 μ M), a mitochondrial uncoupler to assess maximal oxidative capacity. FCCP, a protonophore, was added to collapse the inner membrane gradient, allowing the electron transporter chain (ETC) to function at its maximal rate, and the maximal respiratory capacity was derived by subtracting non-mitochondrial respiration from the FCCP rate. Rotenone A (0.1 μ M) and antimycin A (4 μ M), inhibitors of complex III and I, were added to shut down the ETC function, revealing the non-mitochondrial respiration (**Fig 2.8**). The mitochondrial reserve capacity was calculated by subtracting basal respiration from the maximal respiratory capacity.

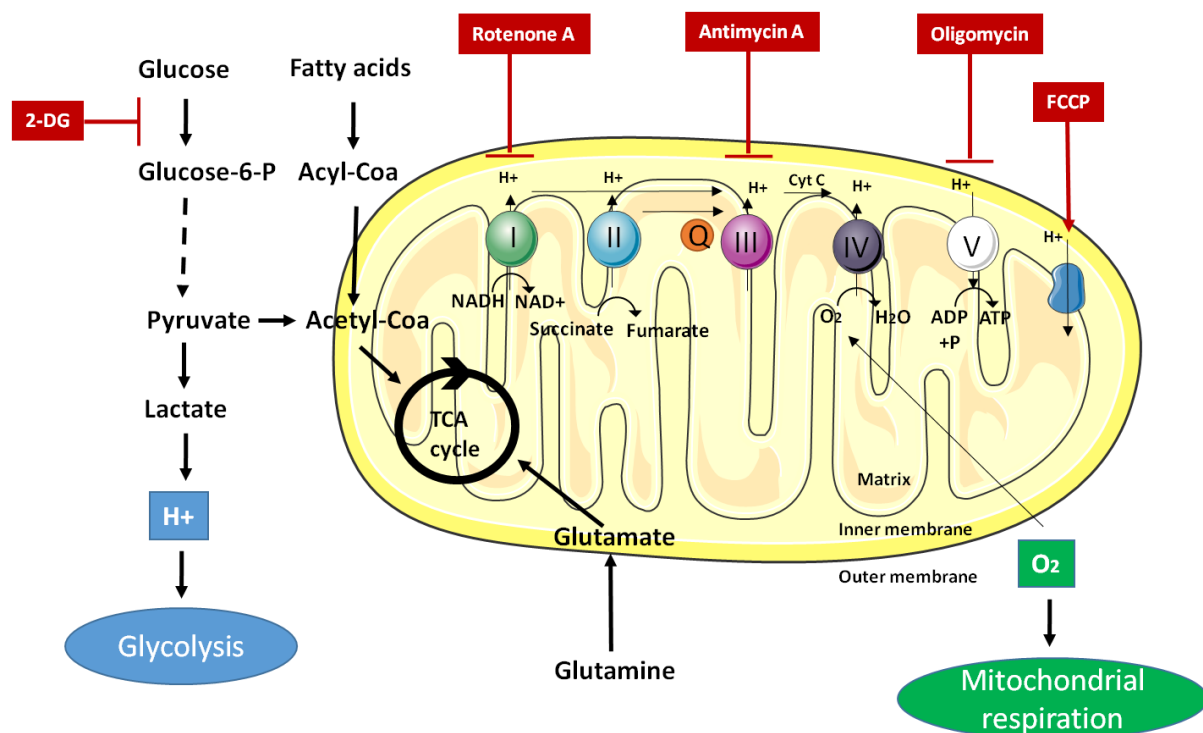


Figure 2.8: Measure of mitochondrial respiration and glycolysis

OCR is measured in order to determine the mitochondrial respiration, and Glycolysis is assessed with H⁺ production.

1.9.2.3. Measurement of ECAR using Extracellular Flux Analyser

The Extra-cellular acidification rate (ECAR) was assessed using an Extracellular Flux (XF) Analyser (Seahorse Biosciences) at the same time as the OCR with the same parameters. Unbuffered DMEM was supplemented with 10mM Glucose for 1h at 37°C in a 21% O₂ incubator before the transfer to the XF24 analyser.

ECAR measurement is based on a measure of acidification (pH) caused by the excretion of lactic acid after its conversion from pyruvate and CO₂ production by the TCA cycle. First, the

glycolytic rate was measured 1h after glucose was supplied. Oligomycin (2 μ M) was then injected. It is an ATP synthase inhibitor in the mitochondrial electron transport chain, which decreases the ATP/ADP ratio and drives glycolysis, enabling a measure of the maximal glycolytic reserve capacity. Lastly, 2-DG (30mM), a glycolysis inhibitor was injected. As its 2-hydroxyl group is replaced by hydrogen, it blocks glycolysis (**Fig 2.8**).

1.9.3. Determination of protein quantification

After the sea-horse experiment, astrocytes were resuspended into 20 μ l of lysis buffer and stored at -80°C. The Thermo Scientific™ Pierce™ BCA Protein Assay, a detergent-compatible formulation based on bicinchoninic acid (BCA) was used for the colorimetric detection and quantitation of total protein. This method combines the reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺¹) using a unique reagent containing BCA. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. A standard curve from 0,125mg to 2mg of BSA was used to determine the protein concentration in each sample. The samples and standard solution were diluted into lysis buffer and 2 μ l of the solution was completed with 16 μ l of working reagent (solution A and B). Protein quantification was determined at 562nm as this water-soluble complex exhibits a strong absorbance at 562nm.

2. In vivo experiments

2.1. Animals

All animal procedures conformed to the European Council Directive 1986 (86/609/EEC) and were approved by the BioResources Ethics Committee of Trinity College Dublin. Male C57BL6/J mice aged 9-14 weeks old (Charles River, UK or Harlan, UK) were kept on a 12h/12h light/dark cycle (lights on from 8.00 to 20.00) with access to food and water *ad libitum*. Mice were singly-housed and handled 5 days prior to behavioural testing.

2.2. Drug preparations

2.2.1. Intraperitoneal administration

All drugs were injected intraperitoneally in an injection volume of 10ml/kg. Concentrations were as follows: ketamine 30mg/kg, TRIM 50mg/kg and ZL006 10mg/kg in 0.9% saline. All

drugs were prepared freshly on the day of testing and were administered after the first exposure of the FST as indicated.

Table 2.7. Drug preparation for IP administration

Compound name	Molecular weight (g/mol)	Injection volume	Concentration	Dissolved in:
Ketamine	238	10ml/kg	30mg/kg	Saline 0.9%
ZL006	328	10ml/kg	10mg/kg	Saline 0.9%
TRIM	212	10ml/kg	50mg/kg	Saline 0.9%

2.2.2. Intra-cortical administration

All injections were made into the pre-limbic cortex (PLC) bilaterally in a 1µl injection volume. Concentrations of agents were as follows: L-AAA 50µg/µl (diluted from stock solution 100µg/µl), Phosphate buffer saline (PBS) 0.1M (1 x PBS) and tetrasodium(6E)-4-amino-6-[[4-[4-[(2E)-2-(8-amino-1-oxo-5,7-disulfonatophthalen-2-ylidene)hydrazinyl]-3-methylphenyl]-2-methylphenyl]hydrazinylidene]-5-oxonaphthalene-1,3-disulfonate (Evans blue) 2% into PBS.

Table 2.8: Drug preparation for intra-cortical administration

Compound name	Molecular weight (g/mol)	Injection volume	Concentration	Dissolved in:
L-AAA	161	1ul bilaterally	100µg/µl	PBS
PBS	-----	1ul bilaterally	0.1M	-----
Evans blue	961	1ul bilaterally	2% into PBS	PBS

2.3. Surgery

2.3.1. Aseptic surgical technique

Aseptic techniques were used in order to prevent any contamination. Before the surgery, all the surfaces and tools were cleaned with Ethanol 70%. All the surgical equipment was cleaned between each operation with Ethanol 70%. The head of the animal was carefully cleaned with Betadine, surgical scrubs.

2.3.2. L-AAA administration in the PLC

Adult mice were anaesthetised by intra-peritoneal (IP) injection using avertin (1.25 g 2,2,2, tribromoethanol; 2.5ml tertiary amyl alcohol; 100ml distilled water) and secured in a stereotactic frame. Before starting the surgery, animals were wrapped in tissue to maintain a normal body temperature. Their heads were shaved surrounding the incision area and

betadine was applied in order to clean the surgical area. EMLA cream was applied directly on the head and ears of the animal. In addition, Lacribule was applied on the eyes to avoid dryness during the surgery.

One injection of L-AAA (50 μ g/ μ l; 1 μ l) or (100 μ g/ μ l; 1 μ l) or two successive injections (50 μ g/ μ l; 1 μ l 24 h apart) were performed bilaterally into the PLC using the following coordinates: 1.7mm anteroposterior, -0.4mm dorsolateral and depth -2.5mm from Bregma (**Fig 2.9**). The same volume of phosphate-buffered saline (PBS 0.1M) was infused into the PLC of the sham-lesioned controls. Treatment with L-AAA or vehicle control (PBS) was infused at a rate of 0.2 μ l/min using a micro-capillary for 5 min. The microcapillary was slowly withdrawn after 2 min. After surgery, mice were wrapped in surgical bandage and placed in an incubator set at 28°C to recover.

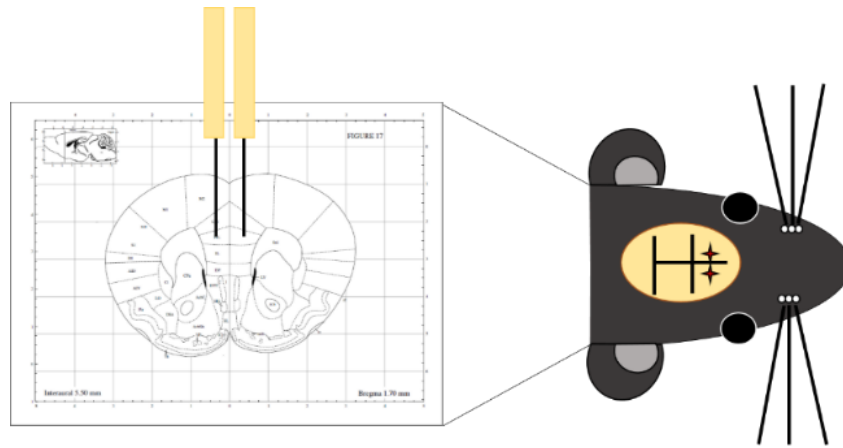


Figure 2.9: Injection site of L-AAA or PBS into the PLC

1 μ l of L-AAA or PBS was infused into the Pre limbic cortex (PLC) using the following coordinates: 1.7mm anteroposterior, -0.4mm dorsolateral, and depth -2.5mm from the Bregma

2.3.3. L-AAA administration in the DG

In a separate experiment, L-AAA (50 μ g/ μ l; 1 μ l; 0.2 μ l/min) or PBS was administered into the DG (2.2mm anteroposterior, -1.4mm dorsolateral and depth -2.1mm from Bregma).

2.3.4. Evans blue injection

Evans blue solution (2% into PBS) was injected using the same parameters as L-AAA or PBS administration bilaterally into the PLC at the following coordinates: 1.7mm anteroposterior, -0.4mm dorsolateral, and depth -2.5mm from the Bregma. The rate of injection was 0.2 μ l per minute for 5 min and the micro-capillary was slowly removed 2 min post injection. Mice were euthanised by decapitation 15 minutes after the end of the surgery while under anaesthesia. Post mortem, the brains were extracted and stored in 30% sucrose solution for 3 days and then snap-frozen using isopentane on dry ice prior to sectioning.

2.4. Behavioural tests

2.4.1. Saccharin preference

Mice were singly-housed in order to carry out saccharin preference testing. Animals were habituated with two 250ml plastic bottles of water in their home cage, for 4 consecutive days. A baseline period was performed with a two-bottle choice test (0.01% saccharin versus water). The location of saccharin and water (Right or Left) was alternated depending on the group of animals but remained the same for the duration of the experiment for each animal. Post-surgical procedures, saccharin and water consumption were then recorded for 3 consecutive days from 9am to examine the effect of L-AAA administration on saccharin preference. Saccharin trials were carried out during the dark and light phases of the cycle to take advantage of the higher levels of consumption during the dark phase (Harkin et al., 2002).

We chose to perform this test with a palatable saccharin solution instead of sucrose solution in order to exclude controversy over the calorific value of sucrose that could impact locomotor activity (Harkin et al., 2002). Saccharin was supplied at a concentration of 0.01%; mice typically display a saccharin preference of approximately 80% at this concentration (Harkin et al., 2002). Fluid consumption was measured by initially weighing the bottles and re-weighing the following morning, and calculating the weight difference (**Fig 2.10**).

$$\% \text{ Saccharin consumption} = \frac{(\text{Weight saccharin D0} - \text{Weight saccharin D1})}{((\text{Weight saccharin D0} - \text{Weight saccharin D1}) + (\text{Weight water D0} - \text{Weight water D1}))} \times 100$$

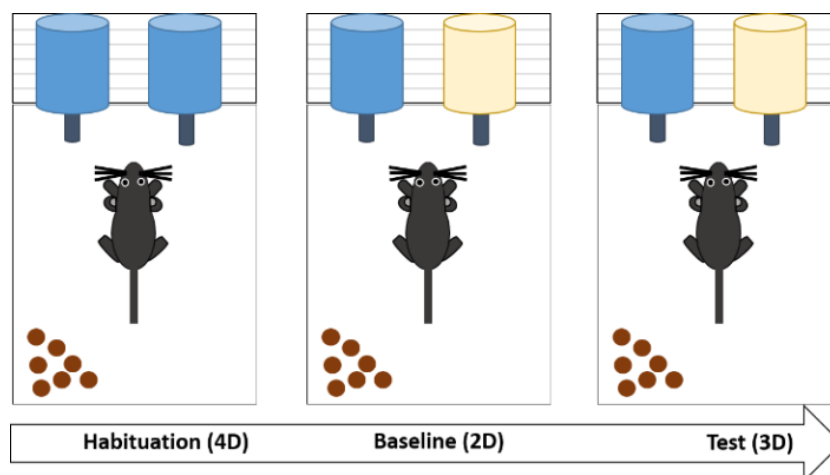


Figure 2.10: Saccharin preference

For 4 days, mice were habituated to 2 bottles of water. Subsequently for 2 days a baseline of saccharin preference was established by presenting a bottle of water and a bottle of saccharin. After surgery, animals were tested for their preference for saccharin over 3 days.

2.4.2. Splash test

It has been reported that the splash test can be used to measure behavioural apathy (Kalueff et al., 2016). This test measures grooming behaviour when the animal is cleaning, licking or scratching its fur following the application of 30% sucrose solution to the dorsal coat of mice in their homecage (Yalcin et al., 2005). The test was carried out 48 h after L-AAA or PBS administration and the duration of grooming was recorded for 10 min after the vaporisation of sucrose solution. The grooming was focused on back licking. Grooming to the head was not included on account of the impact of post-operative recovery from surgery.

2.4.3. Open-field test

OF can be used to assess locomotor activity, exploration and anxiety-like behaviours. The OF consisted of a circular arena (72cm of diameter) with walls at a height of 50 cm and a white background under normal light illumination. The OF test was carried out 24 h, 48 h or 6 days following L-AAA or PBS administration. Before starting the task, mice were placed facing the wall in the peripheral zone. Locomotor activity was assessed by recording the total distance travelled in the arena during the 10 min test. To assess anxiety, thigmotaxis behaviour was characterised by the time spent in periphery (40% of the total arena). Following the OF test, mice were immediately removed from the arena and placed back into their home cage. The arena was cleaned with water and 70% alcohol between each animal in order to remove olfactory cues.

2.4.4. Tail suspension test

The TST was carried out at 48 h, 72 h or 7 days after L-AAA administration. During the TST (**Fig 2.11**), mice were suspended by their tail from a metal rod fixed 35 cm above the surface of a table using adhesive tape attached approximately 0.5 to 1 cm from the base of their tail. Mice were considered immobile only when they hung passively and completely motionless. The total duration of immobility during a 6 min test was calculated. A reduction in immobility in this test indicates an antidepressant-like response. The TST is based on the observation that after a period of time, mice develop an immobile posture when placed in an inescapable stressful situation (Cryan et al., 2005, (Steru et al., 1985)

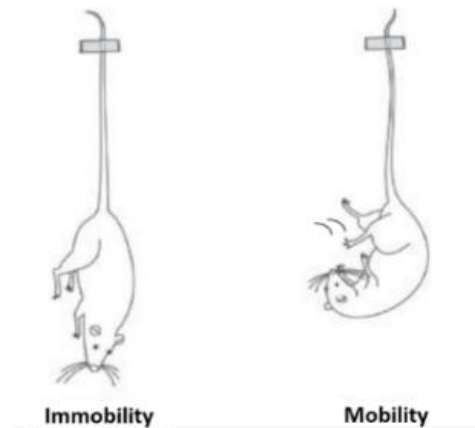


Figure 2.11: TST

For 6 min, animals are hung by their tail and immobility was assessed as a measure of depressive-like behaviour. Animals were considered immobile when they remain passive and completely motionless.

2.4.5. Forced swim test

The FST is sensitive to conventional antidepressant treatment (Porsolt et al., 1977) as well as non-monoaminergic antidepressants (Maeng et al., 2008, Autry et al., 2011).

This test was performed (**Fig 2.12**) as previously described (Porsolt et al., 1977). Mice were placed for 6 min in a 2 litres (l) Pyrex glass beaker filled with 12cm of water at $23\pm 1^{\circ}\text{C}$. Immobility was scored during the last 4 min of the first and second exposure 24 h later. The water in the chamber was changed between animals. A decrease in immobility indicated an antidepressant-like response. Immobility was defined as the absence of active, escape-oriented behaviours such as swimming, jumping, climbing or diving

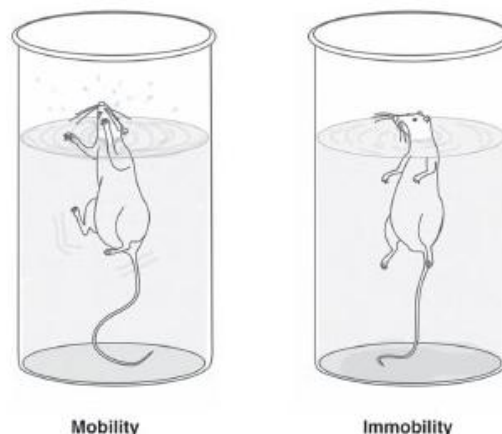


Figure 2.12: FST

For 6 min with a first exposure and a second 24 h later, animals were forced to swim in a 2 litres (l) glass beaker. Immobility was assessed (2-6min) as a measure of depressive-like behaviour. Immobility was considered as an absence of active, escape-oriented behaviours such as swimming, jumping, climbing or diving.

2.5. Post mortem immunohistochemical analysis

2.5.1. Perfusion

Mice were euthanised after behavioural testing at 48 h, 72 h or 7 days after L-AAA administration with Euthanal (0.1ml at 50mg/ml). The descending thoracic aorta was clamped to increase perfusion flow to the head. An incision was made in the left ventricle of the heart where the cannula was inserted. Once perfusion started, the right atrium of the heart was cut to allow drainage. Mice were first transcardially perfused with heparinised PBS (0.1 M, pH 7.4; heparin 1ml at 5000UI/ml) for 3 min, and then with PFA (4% PFA in PBS) for 3 min 30 s. Brains were then removed from the skull and placed in 4% PFA overnight, followed subsequently by placement in 30% sucrose solution (w/v) for 3 days. After these steps, brains were stored in a freezing solution at -80°C until sectioning.

2.5.2. Immunohistochemistry

2.5.2.1. *Glial fibrillary acid protein*

Immunostaining for GFAP is the prototypical method for labelling and imaging astrocytes (Sofroniew, 2009). Following transcardial perfusion, brains were sliced into serial frontal sections (40µm thick) which were immediately transferred to a freezing solution (sucrose 30%w/v, ethylene glycol 30%v/v in PBS) and stored at -80°C. Upon time of use, PLC sections were defrosted and rinsed 3 times for 10 min in PBS and incubated in blocking solution for 40 min at room temperature [10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS]. After this step, slices were rinsed once in PBS and incubated overnight either in negative solution [1% bovine albumin serum (BSA) and 0.1% Triton X-100 in PBS] or in primary antibody solution (rabbit polyclonal anti-GFAP 1:1000, DAKO) at 4°C. Following three 5-min washes in PBS, the slices were incubated with the secondary antibody [1:1000 alexa 488nm fluorescent secondary anti-rabbit (Invitrogen) and 1% BSA in PBS] for 2h at room temperature. The slices were washed 3 times in PBS for 5 min and mounted onto gelatine-coated slides and any excess liquid was removed. Vectashield mounting medium DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until imaged with the confocal microscope.

2.5.2.2. *Calcium-binding protein B*

Immunostaining for S100b is a method for labelling and imaging astrocytes (Sofroniew, 2009). Staining for S100b was performed on frozen slices from the PLC as previously described. Upon time of use, the sections were defrosted and rinsed for 10 min twice in PBS with a last wash in 5% triton diluted into PBS and incubated in blocking solution for 40 min at room temperature [20% normal goat serum (NGS) in PBS]. After this step, the slices were rinsed once in PBS and incubated overnight either in negative solution [1% bovine albumin serum (BSA) and 0.1% Triton X-100 in PBS] or in primary antibody solution [rabbit anti-S100b 1:1000, Abcam, in 1% BSA, 0.1% Triton X-100 in PBS] at 4°C. Following three 5-min washes in PBS, the slices were incubated with the secondary antibody [Alexa 488nm, 1:1000, anti-rabbit (Invitrogen), 1% BSA in PBS] for 2h at room temperature. The slices were washed 3 times in PBS for 5 min and mounted onto gelatine-coated slides and any excess liquid was removed. Vectashield mounting medium DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until imaged with the confocal microscope.

2.5.2.3. *Neuronal nuclear antigen*

NeuN is the prototypical method for labelling and imaging mature neurons (Gusel'nikova and Korzhevskiy, 2015). PLC sections were sliced as previously described, defrosted and rinsed for 10 min 3 times in PBS and incubated in blocking solution for 40 min at room temperature [10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS]. After this step, the slices were rinsed once in PBS and incubated overnight either in negative solution [1% bovine albumin serum (BSA) and 0.1% Triton X-100 in PBS] or in primary antibody solution (mice monoclonal anti-NeuN 1:300, Millipore) at 4°C. Following three 5 min washes in PBS, the slices were incubated with the secondary antibody [1:500 alexa 488nm fluorescent secondary anti-mouse (Invitrogen)] for 2h at room temperature. The slices were washed 3 times in PBS for 5 min and mounted onto gelatine-coated slides and any excess liquid was removed. Vectashield mounting medium DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until imaged with the confocal microscope.

2.5.2.4. *Co-staining of Doublecortin and GFAP*

Immunostaining for DCX is the prototypical method for labelling and imaging immature neurons (Rao and Shetty, 2004) (**Fig 2.13**). In order to confirm that the DCX

immunoreactivity changes were associated with GFAP immunoreactivity impairments in the DG, we performed a co-staining for GFAP and DCX on frozen slices as previously described in the methods for GFAP staining. At time of use, the sections were defrosted and rinsed for 10 min twice in PBS with a last wash for 10 min in 5% triton diluted into PBS and incubated in blocking solution for 40 min at room temperature [20% normal goat serum (NGS) and 5% Triton in PBS]. After this step, the slices were incubated overnight either in negative solution [1% bovine albumin serum (BSA) and 0.1% Triton X-100 in PBS] or with the primary antibody solution (rabbit polyclonal anti-GFAP 1:1000, DAKO and mouse anti DCX, 1:250, Santa-cruz and 1% BSA, 0.1% Triton X-100 in PBS) at 4°C. Following three 5 min washes in PBS, the slices were incubated with the secondary antibody [Alexa 488nm, 1:1000, anti-rabbit (Invitrogen), Alexa 546, 1:1000, Life technologies and 1% BSA in PBS] for 2h at room temperature. The slices were washed 3 times in PBS for 5 min and mounted onto gelatine-coated slides and any excess liquid was removed. Vectashield mounting medium DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until imaged with the confocal microscope.

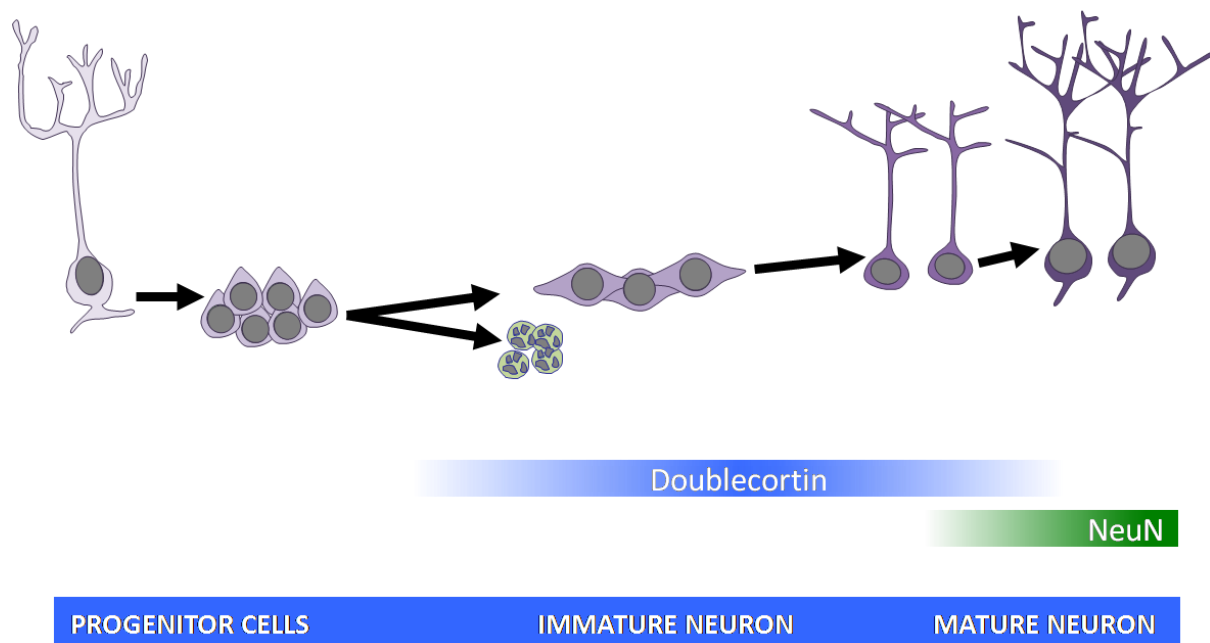


Figure 2.13: Neurogenesis

First, progenitor cells proliferate for 1-3 days leading to either apoptosis or to neuronal differentiation into immature neurons (4-7 days). Immature neurons express DCX and they mature into neurons expressing NeuN (4-6 weeks).

2.5.2.5. *Ionized calcium binding adaptor molecule 1*

Iba1 is a 17-kDa EF-hand protein expressed specifically in microglia and macrophage (Imai et al., 1996). Iba1 has actin-bundling activity and participates in membrane ruffling and phagocytosis in activated microglia (Ohsawa et al., 2004). Immunostaining for Iba1 was used to quantify the microglia number and performed on frozen slices from the PLC obtained as previously described. Upon time of use, sections were defrosted and rinsed 3 times for 10 min in PBS and quenched for 20 min in a hydrogen peroxide solution (1% H₂O₂ and 5% methanol in PBS). After three 5-min washes with PBS, the sections were incubated in blocking solution for 45 min at room temperature [10% normal goat serum (NGS) and 0.3% Triton X-100 in PBS]. After this step, the slices were incubated overnight either in negative solution (5% NGS and 0.05% Triton X-100 in PBS) or with the primary antibody solution (rabbit polyclonal anti-IBA1 1:3000, Vectastain, Elite vector ABC kit; Vector laboratories, 0.05% triton X-100 PBS and 2% of NGS into PBS) at room temperature. On day 2, Following three 5-min washes in PBS, the slices were incubated with the secondary antibody [with 4 drops of biotinylated goat anti rabbit (Vectastain, Elite ABC kit; Vector laboratories, 6 drops of NGS into 40ml of PBS] for 90 minutes in the dark at room temperature. The slices were washed 3 times in PBS for 5 min and incubated for 90 minutes with avidin-biotin complex [3 drops of A and 3 drops of B (Vectastain, Elite ABC kit, Vector laboratories) into 30ml of PBS prepared 30 min in advance]. Following three additional 5-min washes with PBS, the slices were incubated for around 2 min with 3,3'-Diaminobenzidine (DAB solution; 4mg/ml, 2ml of DAB solution for 40ml of PBS with 12µl of H₂O₂). To stop the DAB reaction, the slices were washed for 5 min into distilled water followed by two 5-min washes into PBS. Lastly, the slices were mounted onto gelatine-coated slides and allowed to dry overnight after any excess liquid was removed.

On the third day, the slides were washed into distilled water and dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) for 2 min and finally Xylene for 1 min. DPX mounting media was then placed on the slides and a coverslip carefully placed on top. The slides were then allowed to dry for 24 h before being imaged.

2.6. Image analysis

2.6.1. GFAP quantitative analysis of cells of interest

Image stacks of the regions of interest were acquired at a magnification 100x (10x objective) with a zoom of 2 using the Leica confocal SP8 microscope and FIJI software. Selected

images were then imported into ImageJ for processing and quantification. Image stacks were projected into a single image allowing for the quantification of cells over 30µm. GFAP positive cells were quantified using a method adapted from (Gosselin et al., 2009) (**Fig 2.14**). GFAP immuno-reactive cells were quantified by 3 criteria: (1) Continuous labelling with at least three distinct processes, to prevent quantification of single processes transversally crossing the section; (2) do not cross the limit of the acquired field; (3) are not involved in the formation of a blood vessel, as astrocytes surrounding vessels are often hardly individually distinguishable. The total number of GFAP positive cells in the PLC to meet these criteria was counted in 3 squares of 250µm by 250µm contours per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm².

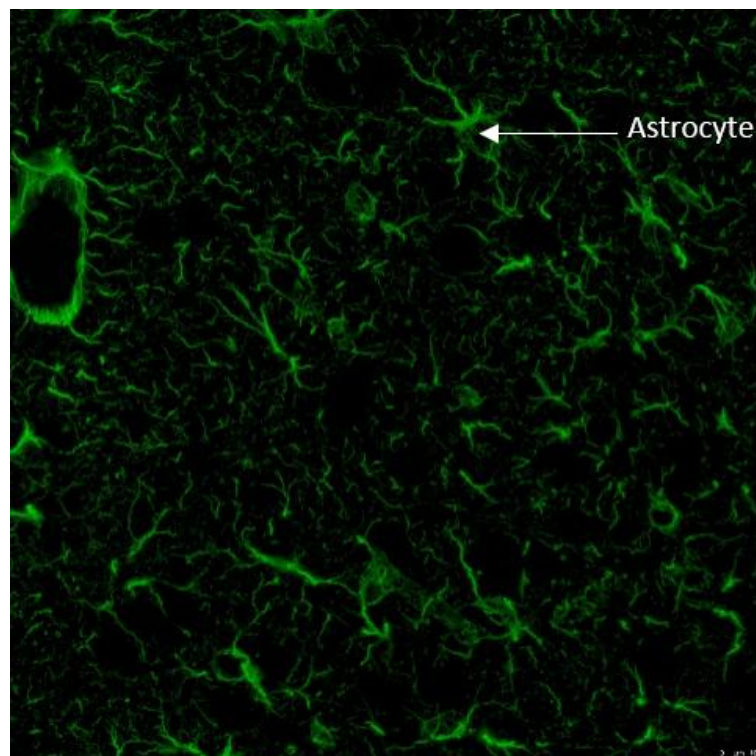


Figure 2.14: Confocal imaging of GFAP in the PLC

GFAP immuno-reactivity (Green on the picture) using 10x objective was used as a measure to quantify the number of astrocytes depending on strict parameters (three distinct processes; do not cross the limit of the acquired field, are not involved in the formation of a blood vessel). Total number of GFAP positive cells was counted in the PLC in 3 squares of 250µm by 250µm per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm². (The representative picture was taken using a 20x objective).

2.6.2. S100b quantitative analysis of cells of interest

Image stacks of the regions of interest were acquired with a magnification 200x (20x objective) using the Leica confocal SP8 microscope with a zoom of 0.7. Quantification for S100b was done in a similar way to the GFAP analysis: the total number of S100b positive cells was counted in the PLC in 3 squares of 250µm by 250µm contours per region in the right and left hemispheres of the brain (**Fig 2.15**). The number of cells was then averaged and expressed as number of cells per mm².

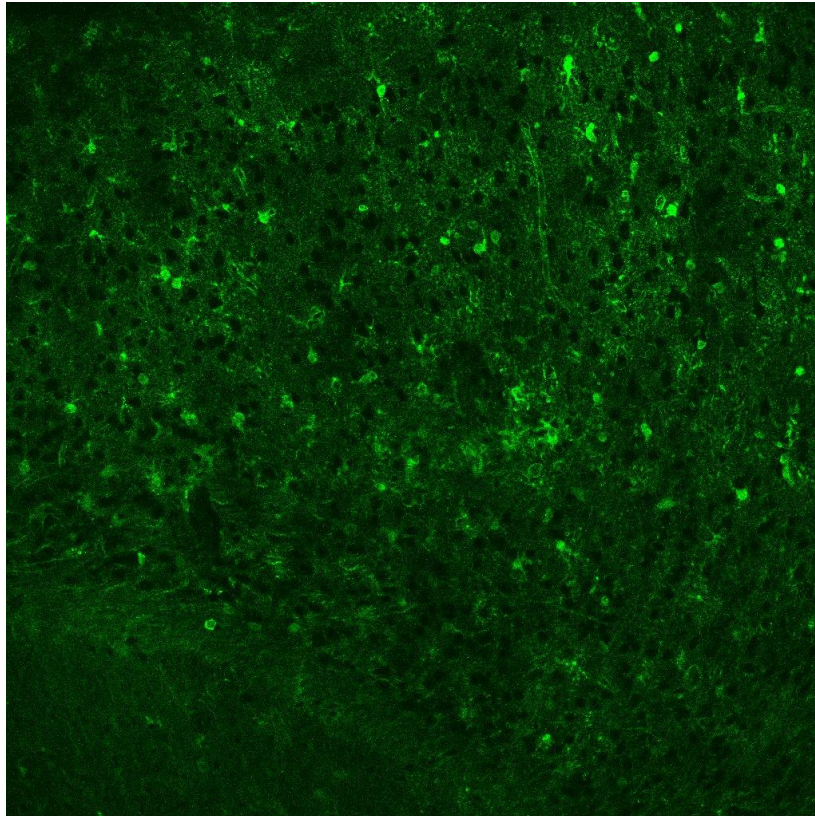


Figure 2.15: Confocal imaging of S100b in the PLC

S100b Immunoreactivity (Green on the picture) using a 20x objective was used to quantify the number of astrocytes. Total number of S100b positive cells was counted in the PLC in 3 squares of 250µm by 250µm per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm²

2.6.3. NeuN analysis of cells of interest

Image stacks of the regions of interest were acquired with a magnification 200x (20x objective) using the Zeiss Axiovert 200M confocal microscope with a zoom of 0.7. Quantification for NeuN was done in a similar way to the GFAP analysis: the total number of NeuN positive cells was counted in the PLC in 3 squares of 250µm by 250µm contours per region in the right and left hemispheres of the brain (**Fig 2.16**). The number of cells was then averaged and expressed as number of cells per mm².

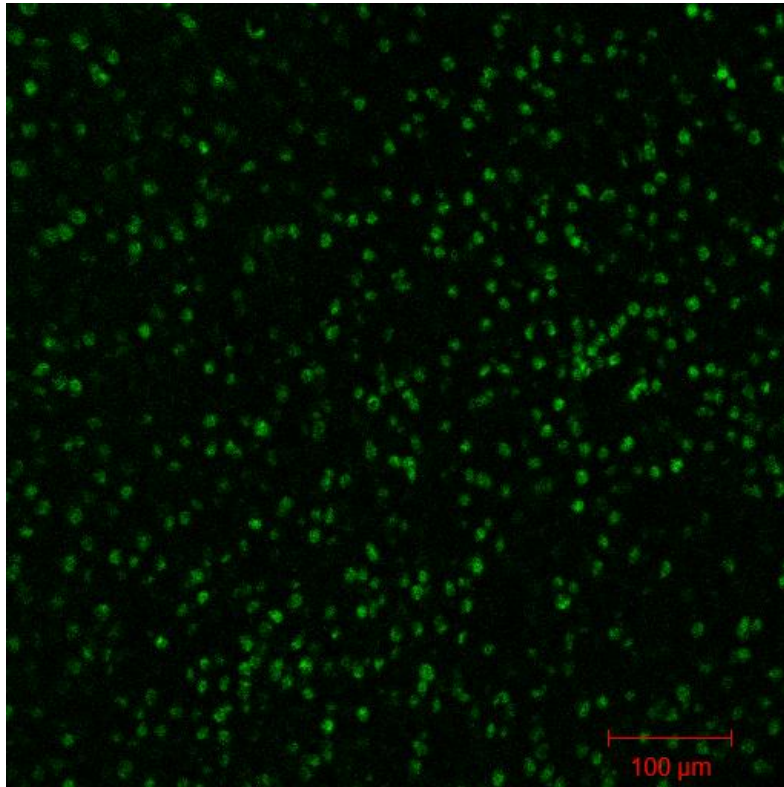


Figure 2.16: Confocal imaging of NeuN in the PLC

NeuN immuno-reactivity (Green on the picture) using a 20x objective was used as a measure to quantify the number of mature neurons. Total number of NeuN positive cells was counted in the PLC in 3 squares of 250μm by 250μm per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm²

2.6.4. DCX/GFAP quantitative analysis of cells of interest

Image stacks of the regions of interest were acquired with a magnification 200x (20x objective) using the Leica confocal SP8 microscope with a zoom of 0.7. GFAP was quantified again in the DG to confirm the changes observed in the PLC. Quantification for GFAP and DCX was done in a similar way to the GFAP analysis: the total number of GFAP or DCX positive cells was counted in the DG in 3 squares of 250μm by 250μm contours per region in the right and left hemispheres of the brain (**Fig 2.17**). The number of cells was then averaged and expressed as number of cells per mm².

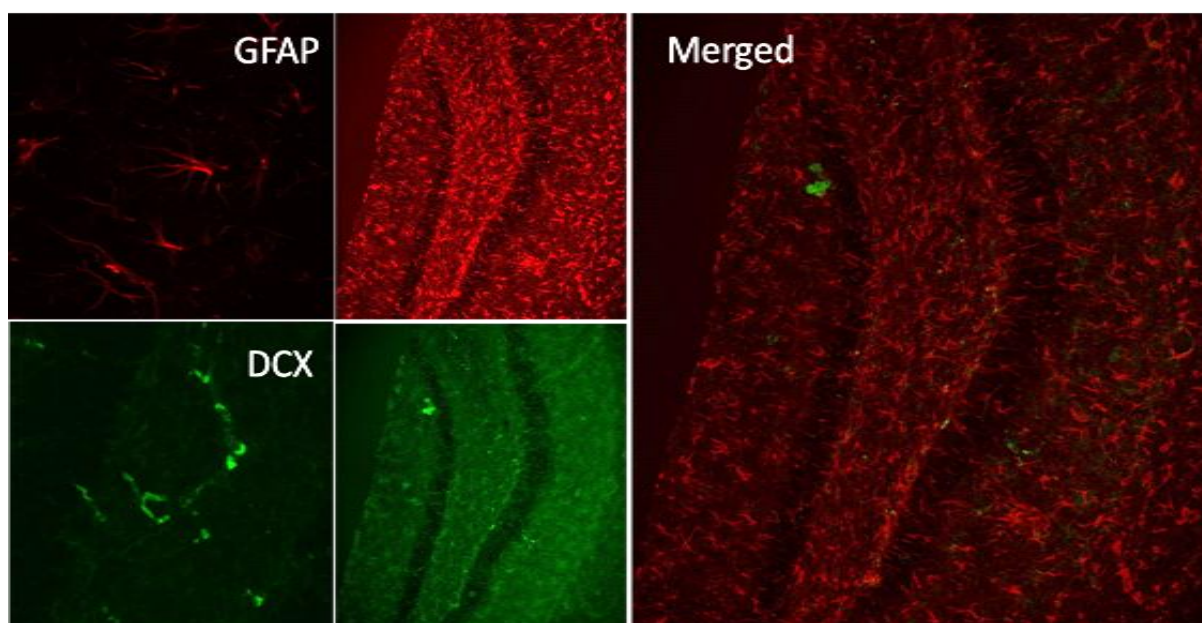


Figure 2.17: Confocal imaging of GFAP and DCX in the DG

GFAP (Red on the picture) and DCX immunoreactivity (Green on the picture) using a 20x objective was used as a measure to quantify the number of astrocytes and immature neurons. Total number of GFAP or DCX positive cells was counted in the PLC in 3 squares of 250 μ m by 250 μ m per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm².

2.6.5. Iba1 quantitative analysis of cells of interest

Image stacks of the regions of interest were acquired with a 200x magnification (20x objective) Olympus bright field microscope. The slides were imaged using the Cell-D software and the total number of microglia in the PLC was estimated in the same way as GFAP+ cells (astrocytes) described previously. Selected images were then imported into ImageJ for processing and quantification. The protocol was adapted and microglia was quantified on 3 different squares of 200 μ m by 200 μ m per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm² (**Fig 2.18**).



Figure 2.18: Imaging of microglia in the PLC

iba1 immuno-reactivity (brown on the picture) using a 20x objective was used as a measure to quantify the number of microglia on 3 different squares of 200µm by 200µm contours per region in the right and left hemispheres of the brain. The number of cells was then averaged and expressed as number of cells per mm².

2.7. Enhanced golgi-cox staining system for dendrites/spine labelling

The “silver” golgi staining, based on the crystallisation of silver chromate ($\text{Ag}^{2+}\text{CrO}_4$) discovered by camillo Golgi in 1873 is one of the main techniques used to study neuronal plasticity. However, the exact mechanism by which individual cells are selectively impregnated still remains unknown. Among the modifications to improve the Golgi staining, the “mercuric” Golgi-Cox method described by Cox in 1891 is widely used with a mixture of potassium dichromate and mercuric chloride instead of silver nitrate. It facilitates the analysis of neuronal morphology and spines through visualization of only a low percentage of neurons (1–3%). Although new techniques including transfection of fluorescent protein [e.g. GFP or yellow fluorescent protein (YFP)] or the use of transgenic animals have emerged, the use of the golgi staining doesn’t require special equipment and allows for the entire brain to be stained at a reduced cost. However, Golgi-staining has some limitations, it takes time and it can be inconsistent (Ranjan and Mallick, 2010). It doesn’t reliably stain long distance axonal projections and fine terminal processes. Therefore, depending on the brain area, it can be difficult to determine the processes that belong to each neuron.

2.7.1. Golgi-cox staining

The superGolgi Kit (Bioenno) is designed for the labelling of dendritic branches and spines of neurons, based on the principle of Golgi-Cox impregnation (Bioenno Tech, Irvine, CA, USA). Mice were transcardially perfused with saline 0.9% solution for 3 min and their brains rinsed and incubated for 10 days into solution A (Impregnation solution) and renewed after 2 days. When the impregnation was ready, the tissue blocks were rinsed with distilled water and transferred into solution B (Post-impregnation buffer: 30g of reagent B in 90ml dH₂O) for 1 day, renewed and kept until use at 4°C.

Using a vibratome, 150µm coronal sections were collected into a B-prepared collection (Collection solution: 30g of reagent B in 500ml dH₂O) and mounted on gelatine subbed slides.

The slides were then washed in 0.01M PBS-Triton for 20-30 min and then place in a diluted solution C in a closed staining jar followed by post-staining.

The slides were placed in reagent D prepared post-staining buffer for 20 min in a dark area, and then washed in 0.01M PBS-Triton, 3 times for 5 min. The slides were rinsed briefly in distilled water and then dehydrated, 4 times in 100% alcohol for 5 min and cleared 3 times in xylene for 5 min. Finally, the slides were coverslipped with DPX and coded to obscure the experimental group of each animal until the final step of statistical analysis.

2.7.2. Golgi-cox spine and primary neurite analysis

Image stacks of the regions of interest were acquired in the PLC at a magnification 200x (20x objective) with an Olympus IX81-long focal length fluorescent microscope to analyse the number of primary neurites emerging from the soma. Z-stacks were acquired for spine analysis were acquired at a magnification 600x (60x objective). 7 apical and 5 basal dendritic segments of 30 µm were acquired per neuron with a number of 5 neurons per animal (3-4 animals per group). Dendritic segments were selected only 1) for non-truncated dendrites, 2) with a dark and uniform staining along all projections and 3) if they were relatively isolated from neighbouring stained neurons. Spine quantification was performed using Neuron studio software and spine morphology was classified as mushroom, thin and stubby. Each individual spine's length and width were measured by the software and, based on these measurements, the spines were classified in the following subtypes: stubby (no neck), mushroom (head diameter: 4µm and neck length <2µm), thin (head diameter smaller than the neck length:

>2 μ M). All values were calculated by averaging the number of spines, divided by the dendritic length of the sample (**Fig 2.19**).

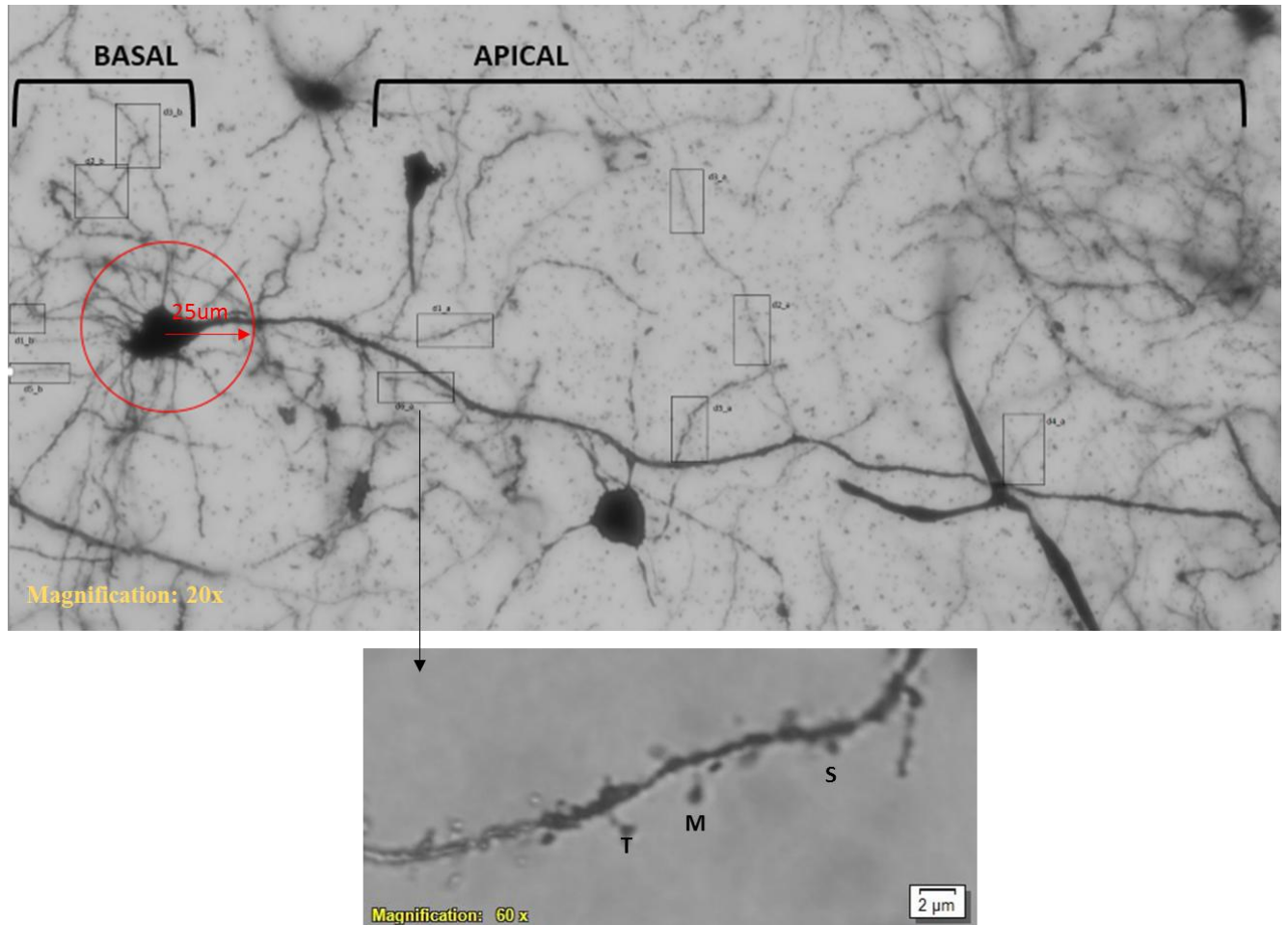


Figure 2.19: Light microscope imaging of golgi-cox stained neurons in the PLC

30 μ m dendritic segments were taken for both apical and basal dendrites. Spine quantification was performed using Neuron studio software. Spine morphology was classified as mushroom, thin and stubby at a 60x objective (T: thin, M: mushroom, S: stubby).

2.8. Statistical analysis

Data are expressed as group means with standard errors (SEM) and were analysed using unpaired Student's *t* tests or analyses of variance (ANOVA). If any statistically significant change was found following one or two factors ANOVA, post-hoc comparisons were performed using Fisher's LSD. Data were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). Analysis was carried out with Graphpad prism 6.

Chapter 3

Inhibitors of the NMDA-R/NO signalling pathway rescue neuronal atrophy and synapse loss provoked by L-AAA-treated astrocytes

1. Introduction

Astrocytes are the most abundant cell type in the CNS and play crucial roles for the maintenance of normal brain function such as the regulation of (1) brain metabolism (2) glutamate transmission (3) neuronal outgrowth and (4) dendritic morphology (Matsutani and Yamamoto, 1998; Dijkstra et al., 1999; Johansson and Strömberg, 2002; Previtera et al., 2010; Sofroniew and Vinters, 2010). Astrocytes are known to regulate neuronal morphology through the provision of extracellular matrix (ECM) molecules which form a permissive substrate to encourage neuronal guidance and development (Jiang et al., 2007; Previtera et al., 2010). In addition, astrocytes actively secrete soluble factors which enhance neurite growth and provide neuroprotection against toxic stimuli (Previtera et al., 2010; Lu et al., 2015; Takemoto et al., 2015). The effect of these secreted factors can be assessed *in vitro* through the collection of cultured media from astrocytes – termed 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 ROS and RNS (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 neurotrophic factors from astrocytes including fibroblast growth factor 2 (FGF-2), GDNF, IL-6, BDNF and nerve growth factor β (NGF- β) (Day et al., 2014).

In addition to providing neuroprotection, there is compelling evidence that astrocytes are involved in regulating synapse formation and function both *in vitro* and *in vivo* (Ullian et al., 2004; Eroglu and Barres, 2010; Allen, 2013; Chung et al., 2013, Chung et al. 2015, Papouin et al. 2017). *In vitro* studies demonstrated that cultured retinal ganglion cells (RGCs) form few synapses in the absence of astrocytes (Pfrieger and Barres, 1997), while astrocyte CM induces synaptogenesis (Nägler et al., 2001), suggesting that synaptogenic effects are mediated through the secretion of soluble factors rather than direct contact-mediated signalling exclusively. These soluble factors have been found to include the ECM proteins thrombospondin (TSP) (Christopherson et al., 2005), cholesterol (Mauch et al., 2001; Pfrieger, 2003) and TNF α (Beattie et al., 2002). One study demonstrated an important role for astrocytes in synapse elimination in both the developing and adult brain (Chung et al., 2013), suggesting that astrocytes act as important modulators of synaptic plasticity throughout life. The re-evaluation of the role of astrocytes in dendrite morphology and synapse formation reflects increasing literature linking astrocytic dysfunction with neuronal

atrophy and synaptic dysconnectivity implicated in several disease states including Alzheimer's and Parkinson's disease (Maragakis and Rothstein, 2006; Verkhratsky 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). Astrocyte-mediated toxicity involves several soluble factors; cytokines (Falsig et al., 2004; Buffo et al., 2010; Garwood et al., 2011); lipocalin 2 (Bi et al., 2013) components of the complement system (Pekny et al., 2007; Bodea et al., 2014) or changes due to altered ATPase expression (Gallardo et al., 2014). Some small molecules with cell death-inducing properties could also play a role in astrocyte-mediated toxicity: the glycosphingolipid–lactosylceramide (LacCer) (Mayo et al., 2014), the ganglioside GD3 (Simon et al., 2002) or their basic core component, the pro-apoptotic mediator ceramide (Wang et al., 2012) and chondroitin sulfate proteoglycan, which is secreted by astrocytes and impedes axon regeneration (Silver and Miller, 2004).

The selective astrocyte toxin L-AAA has received particular attention in recent years as an effective method of selectively inducing impaired astrocytic function. L-AAA is a glutamate analogue occurring 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 indicate that L-AAA acts by inhibiting glutamate transporters and blocking the astrocyte specific enzymes glutamine synthetase (GS) and γ -glutamylcysteine synthetase (Olney et al., 1980; McBean, 1994; Brown and Kretzschma, 1998). More recent data has shown that prolonged exposure (>72 h) of astrocytes to L-AAA (1mM) induces apoptosis through the inhibition of protein synthesis (Nishimura et al., 2000). Recently, L-AAA has been used *in vivo* (Banasr and Duman, 2008; Lee et al., 2013; Liu F., et al., 2017). L-AAA-induced astrocytic ablation in the prelimbic cortex (PLC) is sufficient to induce depressive-like behaviours in rodents, supporting the hypothesis that astrocyte loss contributes to the pathophysiology underlying depressive symptoms (Banasr and Duman, 2008; Lee et al., 2013). A recent study in rats also demonstrated that L-AAA-induced astrocytic dysfunction predisposes to neuronal loss with evidence of decreased NeuN immunoreactivity and dendritic atrophy following delivery of L-AAA to the mPFC (Lima et al., 2014).

Additionally, astrocytes appear to play a key role in providing neuronal protection by regulating extracellular levels of glutamate (Seifert et al., 2006b). Taken into consideration the role of astrocytes in glutamate regulation, dysfunctional astrocytes could contribute to abnormal glutamate concentrations with potential knock-on effects on neuronal plasticity [For review, (Wang et al., 2017)]. Glutamate at physiological concentrations acts as a permissive cue for outgrowth, whereas glutamate excess predisposes to neuronal atrophy (Mattson et al., 1988; Metzger et al., 1998; Esquenazi et al., 2002; Podestá et al., 2014), dendrite regression and spine loss (Monnerie et al., 2003).

Modulators of the NMDA-R in particular have been investigated for their therapeutic potential in the treatment of stroke, epilepsy, TBI, neuropathic pain, migraine, schizophrenia and major depression (Ikonomidou and Turski, 2002; Konradi and Heckers, 2003; Muir, 2006; Maeng and Zarate, 2007; Zhou et al., 2010; Lauritsen et al., 2016). The NMDA-R antagonist ketamine has been shown to rapidly increase the expression of synaptic proteins and spine number in the PFC in the CMS model of depression in rats (Li et al., 2011; Duman and Li, 2012). Ketamine induces changes in PFC spines/synapses rapidly within 24 h of administration whereas conventional antidepressants require chronic administration. IC87201 and ZL006, two small-molecule inhibitors of the PSD-95/nNOS interface have been shown to promote differentiation of neural stem cells (NSCs), neurite outgrowth and dendritic spine formation in the rat MCAO model of cerebral ischemia (Luo et al., 2014). In addition, previous investigations from our laboratory found that ketamine, inhibitors of the PSD-95/nNOS interaction IC87201 and ZL006 and the nNOS inhibitor TRIM possess the ability to attenuate NMDA-induced neurite retraction in rat primary cortical neurons (M.V. Doucet et al., 2015). ZL006 exerts anti-depressant properties in the FST in Wistar Kyoto rats (Sherwin et al., 2017b) while both IC87201 and ZL006 elicit antidepressant-like properties in mice in keeping with putative novel neurotrophic properties of these compounds (Doucet et al., 2013).

2. Aims

Given the key roles that astrocytes play in maintaining neuronal integrity and complexity, synapse formation, plasticity, and function, we sought to investigate the effect of both healthy astrocytes and dysfunctional astrocytes (following L-AAA treatment) on neurite outgrowth and synapse formation using an *in vitro* model consisting of astrocyte-CM. One of the advantages of this model is that the use of astrocyte CM permits investigation of the effect of soluble factors released from astrocytes in isolation from direct interactions.

In this investigation, we aimed to firstly determine the effect of L-AAA on the viability of astrocytes and neurons and to further assess the effect of L-AAA on astrocytes by quantifying the expression of the phenotypic marker GFAP and glutamate transporter GLAST in addition to its effects on astrocytic mitochondrial respiration and glycolysis.

Following on from this, the effect of L-AAA-CM taken from L-AAA-treated astrocytes was assessed on the complexity of primary cortical neurons and formation of synapses.

Finally, we examined the effect of inhibitors of the neuronal NMDA-R/NO signalling pathway as a strategy to prevent atrophic effects following L-AAA-CM induced neuronal atrophy based on reports indicating that low concentrations of NMDA-R antagonists possess protective properties (Robson et al., 2012; Brown et al., 2015; Doucet et al., 2015).

3. Study design

3.1. Effects of astrocyte CM on neurite outgrowth

Primary mixed glia cells were cultured until confluent (DIV 10) before astrocyte isolation. Purified astrocytes (DIV 13) were provided with fresh media for 24 h and the resulting media (CM) was applied to primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry for Sholl analysis (**Fig 3.1**).

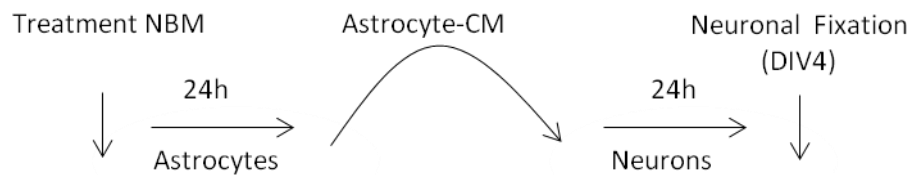


Figure 3.1: Experimental timeline for determination of the effect of astrocyte CM on neuronal complexity

The resulting conditioned media (CM) from astrocytes (termed CM) or control neurobasal media (NBM) was transferred onto primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry for Sholl analysis.

3.2. Effects of direct application of L-AAA and astrocyte L-AAA-CM on neurite outgrowth

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.5mM) for 24 h and the resulting L-AAA CM was applied to primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry for Sholl analysis. 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 (**Fig 3.2**).

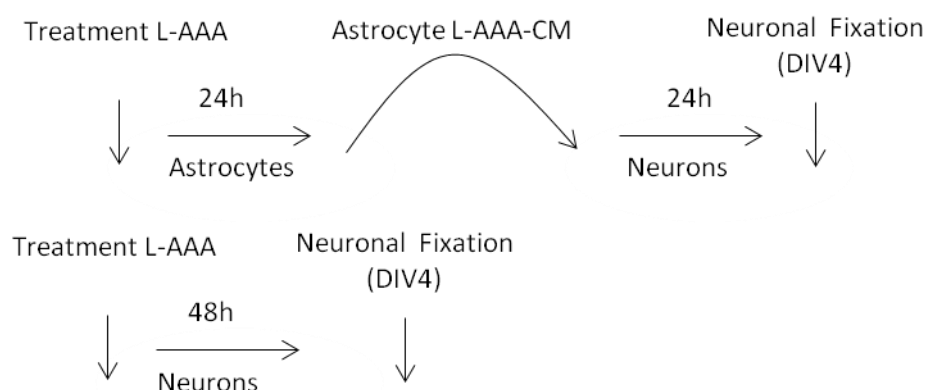


Figure 3.2: Experimental timeline for determination of the effects of astrocyte L-AAA-CM or L-AAA on neuronal complexity

The resulting L-AAA CM from astrocytes (termed L-AAA CM) or control CM was transferred onto primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry for Sholl analysis. In another experiment, L-AAA or NBM was directly applied for 48 h onto primary cortical neurons (DIV 3) before fixation and immunocytochemistry for Sholl analysis.

3.3. Effect of astrocyte L-AAA CM on dendritic spine density in GFP transfected hippocampal neurons

Hippocampal neurons were transfected at DIV7 with lipofectamine 2000 (LF2000) and plasmid incorporating GFP DNA (University of Turku, Finland). Cells were treated at DIV15 for 24 h with L-AAA-CM from cortical astrocytes (0.05mM) or control CM and fixed with PFA. Neurons were visualised with a 40x objective using the Leica confocal SP8 microscope with a zoom selection of the dendritic segment of interest. Analysis was performed on apical and basal dendritic spine density (for further details please refer to chapter 2 material and methods).

A pilot study on GFP DNA transfection was set up to analyse the effect of L-AAA CM on dendritic spine density in hippocampal neurons. However, the study didn't quantify neuronal complexity in hippocampal neurons as hippocampal and cortical neurons are different. This investigation aimed to assess spine density and neuronal complexity in the GFP DNA transfected cortical neurons; however the neurons died shortly after transfection. The protocol in cortical neurons could be adjusted with a different culture media that used for hippocampal neurons in future trials and the volume of culture media or treatment could be increased to preserve the neurons following transfection.

3.4. Effects of L-AAA on viability of cortical primary neuronal and glial cultures

The specificity of L-AAA as an astroglial toxin was investigated by assessing the effects of L-AAA (0.5mM) on astrocytes and neurons *in vitro*. Primary cortical purified astrocytes

(DIV 13) and neurons (DIV 3) were treated with L-AAA for 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.

3.5. Effects of L-AAA on mRNA expression of GFAP and GLAST in astrocytes

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.5mM) for 24 h. Cells were harvested for RNA extraction followed by RT-PCR. mRNA expression of the astroglial markers, GFAP, S100b, the glutamate transporter GLAST and the apoptotic marker caspase 3 was quantified.

3.6. Effects of L-AAA on astrocytic mitochondrial respiration and glycolysis

Primary cortical astrocytes (DIV 14) were treated with L-AAA (0.5mM) for 24 h and the OCR and ECAR were assessed as an indicator of mitochondrial respiration, cellular function and glycolysis using the Extracellular Flux Analyzer (Seahorse Biosciences).

3.7. Effects of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reductions in neuronal complexity

Inhibitors of the NMDA-R/NO signalling pathway were assessed to determine whether they might protect neurons from L-AAA CM-induced neurite regression. Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.5mM) for 24 h. Primary cortical neurons (DIV 3) were co-treated with L-AAA CM in addition to ketamine (100nM), IC87201 (100nM), TRIM (100nM) or ZL006 (100nM) 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 IC_{50} of 2.7 μ M (Florio et al., 2009). For ZL006, the concentrations were selected on the basis that ZL006 has previously shown to inhibit NMDA-induced PSD-95/nNOS binding in cortical neurons (DIV 7-9) with an IC_{50} of 82nM following treatment with glutamate (50 μ M) and glycine (10 μ M) for 30 min (Zhou et al., 2010). In addition, treatment with the NMDA-R antagonist ketamine and MK-801 (10nM) in addition to TRIM (100nM) counteracted the NMDA/glycine-induced reduction in neurite outgrowth. Similar

effects were obtained with IC87201 (10 and 100nM) and ZL006 (10 and 100nM) (M.V. Doucet et al., 2015). (**Fig 3.3**).

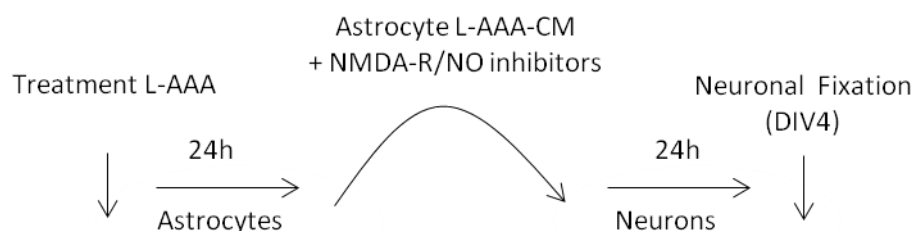


Figure 3.3: Experimental timeline for determination of the effects of NMDA-R/NO signalling inhibitors on neuronal complexity of neurons treated with astrocyte L-AAA-CM

The resulting L-AAA CM from astrocytes (termed L-AAA CM) or control CM was transferred onto primary cortical neurons (DIV 3) in the absence/presence of drugs (Ketamine, TRIM, IC87201, ZL006 each at 100nM) for 24 h before fixation and immunocytochemistry for Sholl analysis.

3.8. Effects of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reductions in the co-localised expression of synaptophysin and PSD-95

Primary cortical astrocytes (DIV 13) were treated with L-AAA (0.05 or 0.5mM) for 24 h and the resulting CM from these astrocytes was applied to mature primary cortical neurons (DIV 20) for 24 h in the presence/absence of ketamine, TRIM or IC87201 and ZL006 (each at 100nM) before fixation and immunocytochemistry for quantification of the number of synaptic puncta comprising co-localised synaptophysin and PSD-95 as described in Chapter 2: Materials and Methods (**Fig 3.4**).

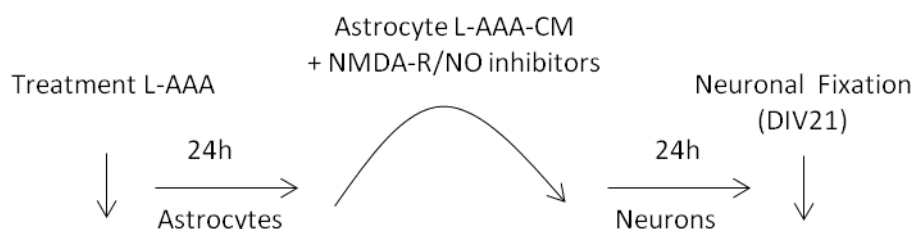


Figure 3.4: Experimental timeline for determination of the effects of NMDA-R/NO signalling inhibitors on synapse formation of neurons treated with astrocyte L-AAA-CM

The resulting L-AAA CM from astrocytes (termed L-AAA CM) or control CM was transferred onto primary cortical neurons (DIV 20) in the absence/presence of drugs (Ketamine, TRIM, IC87201, ZL006, each at 100nM) for 24 h before fixation and immunocytochemistry for Sholl analysis.

4. Statistical analysis

Data are expressed as group means with standard errors (SEM) and were analysed using unpaired Student's *t* tests or analyses of variance (ANOVA). If any statistically significant

change was found following one or two factor ANOVA, post-hoc comparisons were performed using Fisher's LSD. For Student's *t* test, data were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). Because of variations associated with cell cultures data, for Fisher's LSD, a strict threshold was applied, data were deemed significant when $P < 0.01$ (*) or $P < 0.001$ (**). For the data on hippocampal neuronal transfection, normal threshold was applied: $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). Analyses were carried out using GraphPad Prism version 6.

5. Results

5.1. Effect of 24 h exposure to astrocytic CM on neurite outgrowth

Since astrocytes have been shown to promote neuronal growth (Previtera et al., 2010), and CM derived from astrocytes has 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. Treatment of primary cortical neurons for 24 h with CM from astrocytes increased neuronal complexity. Student's T-test showed that CM increased neuritic branches [$T_{(41)} = 2.041$, $P = 0.047$] and number of primary neurites [$T_{(36)} = 3.837$, $P < 0.001$] with an apparent but non-significant increase in neuritic length [$T_{(36)} = 1.310$, $P = 0.198$] when compared to control cNBM (**Fig 3.5A-C**). Two-way repeated measures ANOVA of the number of intersections showed an effect of CM [$F_{(1, 33)} = 11.03$, $p = 0.002$], an effect of distance [$F_{(19, 627)} = 70.51$, $p < 0.001$] and a CM x distance interaction [$F_{(19, 627)} = 3.89$, $p < 0.001$]. Post hoc comparisons revealed that neurons treated with CM had significantly more branches than control-treated neurons at 20-50 μm [$P < 0.001$] and 60 μm [$P < 0.01$] (**Fig 3.5D**).

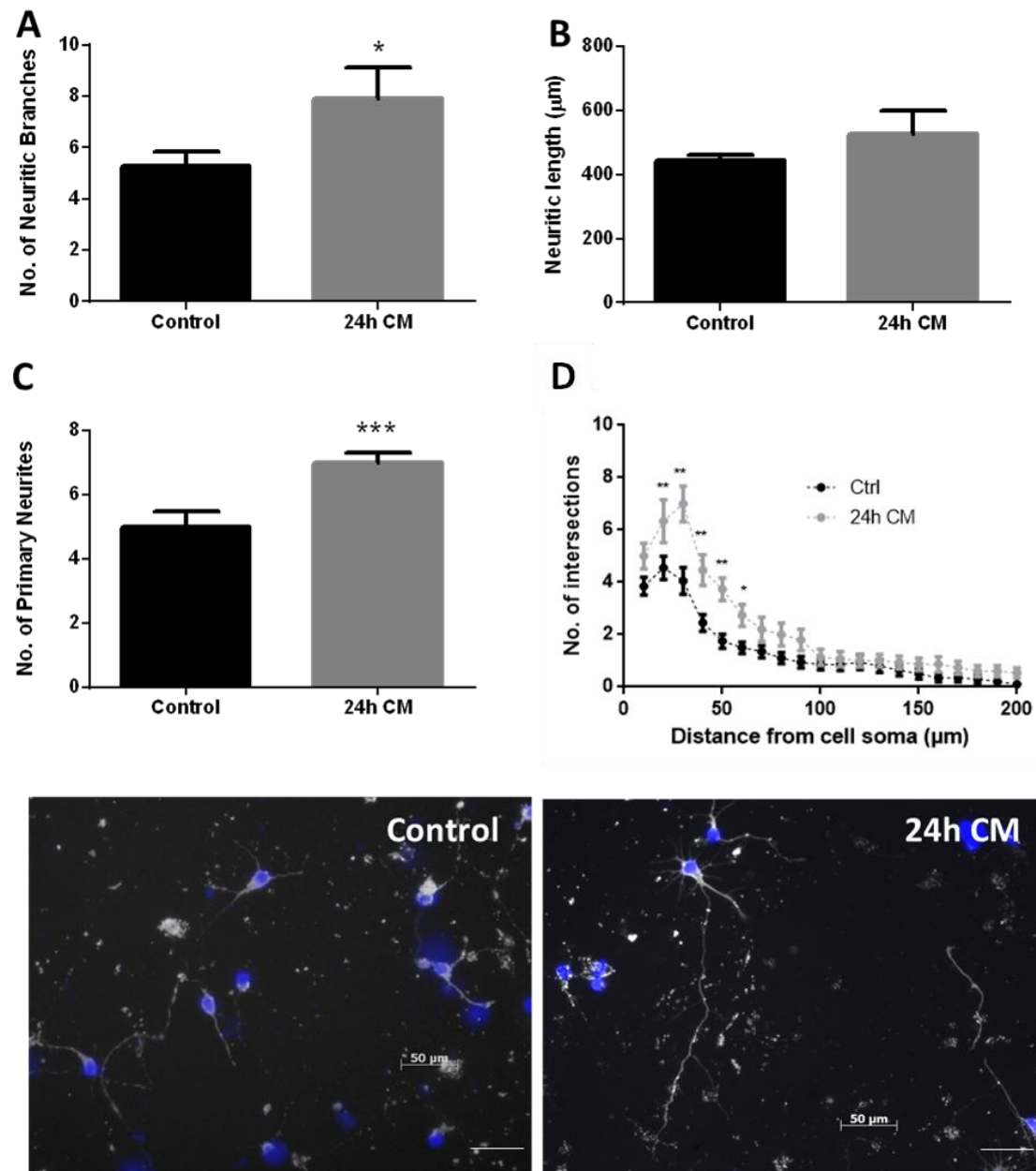


Figure 3.5: Effect of astrocyte CM on neurite outgrowth

Astrocytes (DIV 13) were provided with fresh culture media for 24 h and the resulting CM was transferred to primary cortical neurons (DIV 3) for 24 h before fixation and immunocytochemistry. Neurons were stained for B-III tubulin and DAPI. Sholl analysis was performed on neurons to examine the number of neuritic branches A), the neuritic length B), the number of primary neurites C) and the Sholl profile D). Data are expressed as mean ± SEM, n=15-23 coverslips per group representative of 3 independent experiments. Student's *t* test: ****P*<0.001 ***P*<0.01 **P*<0.05 vs. control; Post hoc comparison ***P*<0.001 **P*<0.01 vs. control. Scale 50μm.

5.2. Effect of 24 h exposure to astrocytic 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 with L-AAA and the media from these cells (24 h L-AAA CM) was

transferred to primary cortical neurons (DIV 3) for 24 h. Treatment of primary cortical neurons for 24 h with CM drawn from L-AAA treated astrocytes decreased neuronal complexity. Student's T-test showed that L-AAA CM decreased the neuritic branches [T (45) =2.828, P=0.007], neuritic length [T (45) =3.327, P=0.0018] and the number of primary neurites 24 h after treatment [T (45) =2.140, P=0.037] when compared to control CM (**Fig 3.6A-C**). Two-way repeated measures ANOVA of the number of intersections showed an effect of L-AAA-CM [F (1,46) =7.986, p=0.007], an effect of distance [F (19,874) =740.4, p<0.001] and a L-AAA CM x distance interaction [F (19,874) =7.088, p<0.001]. Post hoc analysis revealed that neurons treated with L-AAA CM had significantly less branches than control-treated neurons at 10 μ m [P<0.01] and at 20-40 μ m [P<0.001] (**Fig 3.6D**).

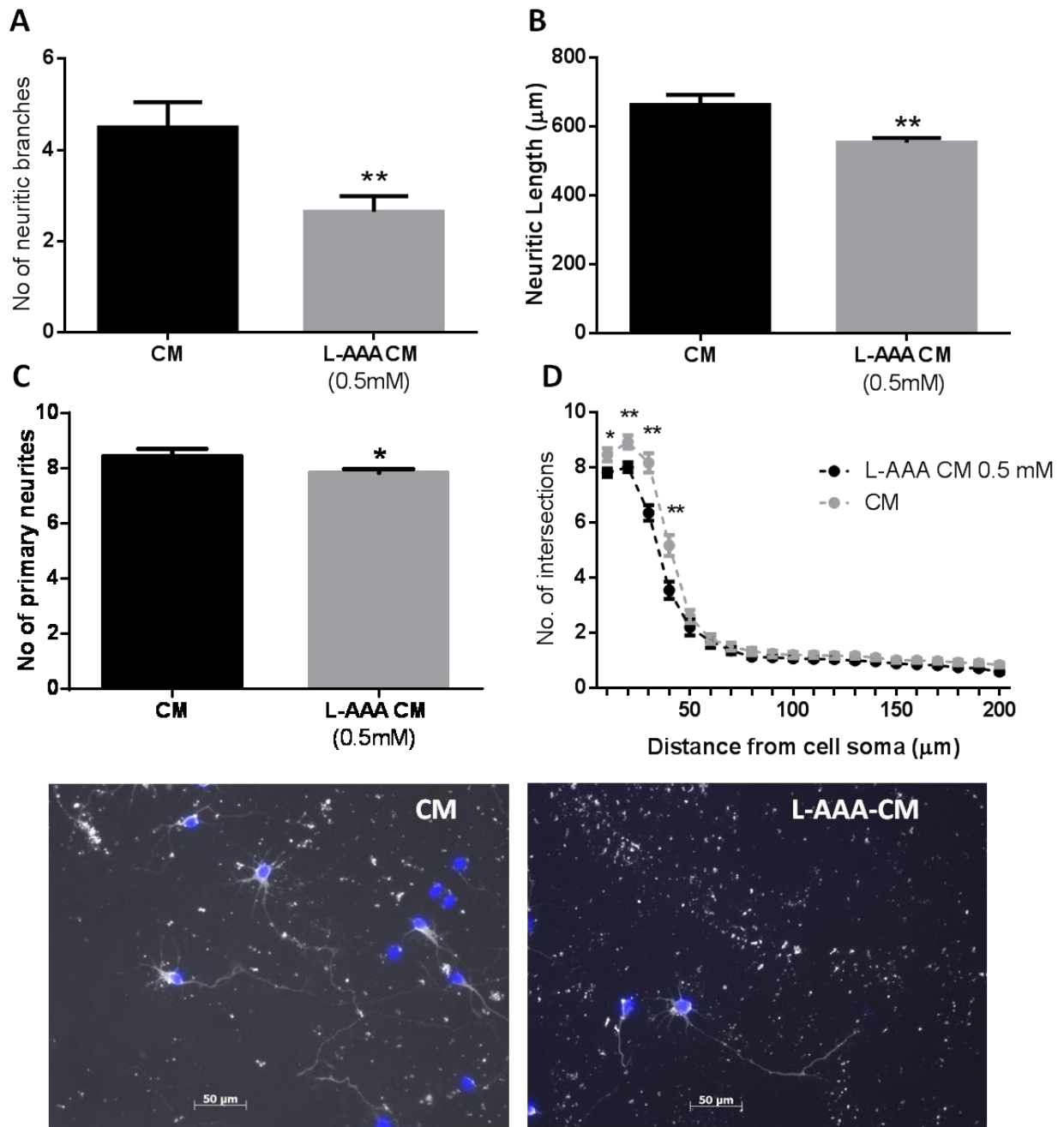


Figure 3.6: Effect of astrocytic L-AAA CM on neurite outgrowth

Astrocytes (DIV 13) were provided with fresh culture media for 24 h with L-AAA (0.5mM) and the resulting astrocyte CM or control 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 neuritic branches A), the neuritic length, B) the number of primary neurites C) and the Sholl profile D). Data are expressed as mean $\pm$ SEM, n=24 coverslips per group representative of 3 independent experiments. Student's *t* test: ****P*<0.001 ***P*<0.01 **P*<0.05 vs. control; Post hoc comparison ***P*<0.001 **P*<0.01 vs. control. Scale 50 μm .

5.3. Effect of 48 h of direct application of L-AAA on neuronal complexity and viability of astrocytes and neurons

L-AAA (0.5mM) was applied to primary cortical astrocytic and neuronal cultures and cell viability and neuronal complexity was determined 48 h later. There was no effect of direct

application of L-AAA on the complexity of neurons; neuritic branches [$T_{(26)}=0.3192$, $P=0.75$], neuritic length [$T_{(26)}=0.5065$, $P=0.616$] or number of primary neurites [$T_{(26)}=0.21$, $P=0.83$] when compared to control cNBM (**Fig 3.7A-C**). Two-way repeated measure ANOVA of the number intersections showed no effect of L-AAA treatment [$F_{(1,26)}=0.2576$, $p=0.6160$], an effect of distance [$F_{(25,650)}=298.5$, $p<0.001$], but no distance x L-AAA interaction [$F_{(25,650)}=0.3751$, $p=0.997$] (**Fig 3.7D**). There was no effect of L-AAA application on neuronal [$T_{(34)}=0.4457$, $P=0.658$] or astrocytic [$T_{(29)}=0.177$, $P=0.86$] viability when compared to control cNBM or cDMEM (**Fig 3.7E-F**) [Student's T-test].

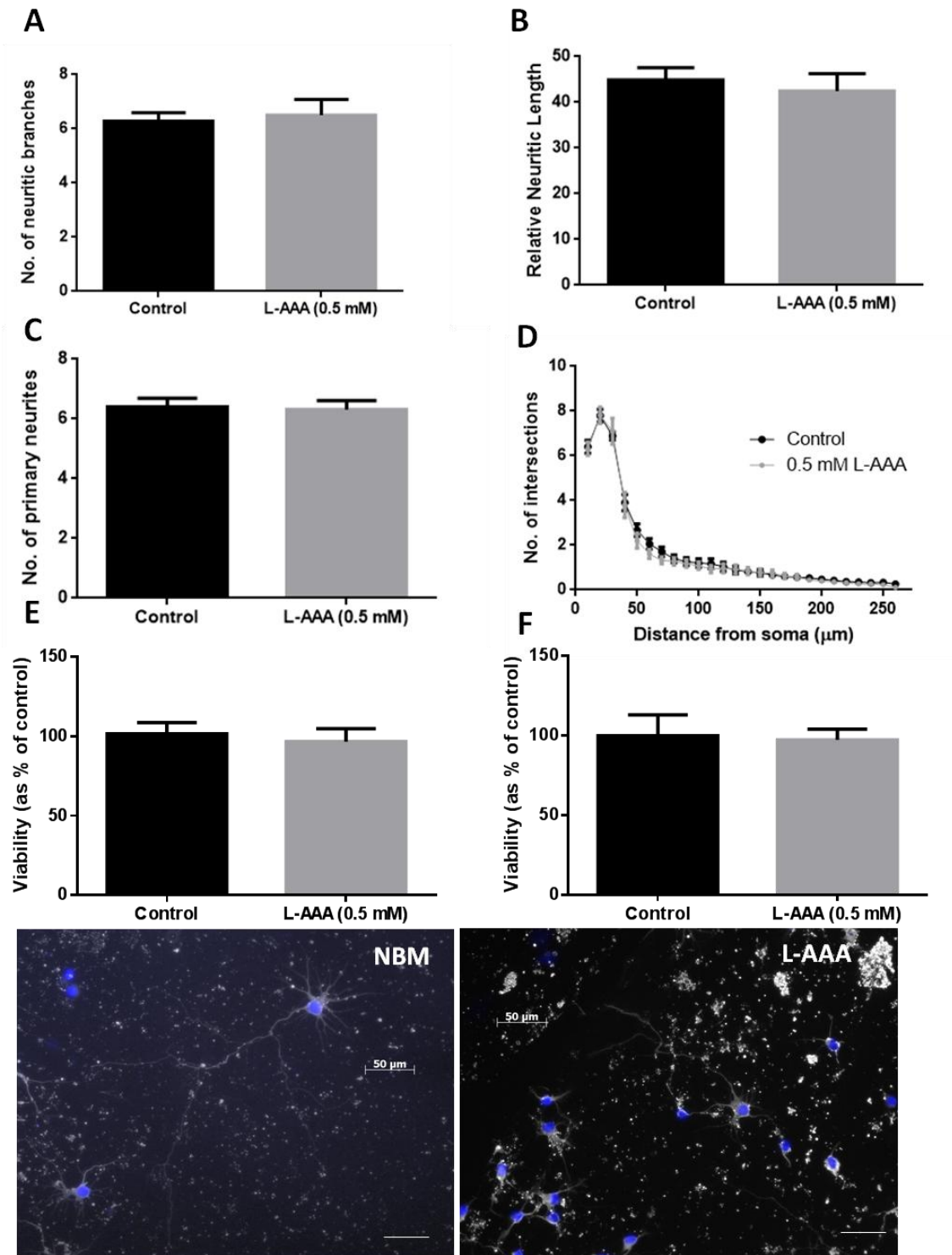


Figure 3.7: Effect of 48 h direct application of L-AAA on neuronal complexity and neuronal and astrocytes viability

Primary cortical neurons (DIV 3) were treated for 48 h with L-AAA (0.5mM) 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 neuritic branches A), the neuritic length B), the number of primary neurites C) and the Sholl profile D). Purified neuronal cultures (DIV 3) E) and primary purified astrocytes were treated at DIV 13 F) were treated with L-AAA for 48 h. Viability was assessed using the Alamar blue assay. Data is expressed as mean $\pm$ SEM, n=15-24 wells representative of 3 independent experiments. Student's *t* test: ****P*<0.001 ***P*<0.01 **P*<0.05. Scale 50 μ m.

5.4. Effect of L-AAA-treated astrocytic CM on spine density in primary hippocampal neurons

The effect of L-AAA on spine density was determined *in vitro* on primary hippocampal neurons. A two-way ANOVA of spine density on apical dendrites showed an effect of L-AAA CM [$F_{(1,21)} = 17.93$, $P < 0.001$], an effect of spine subtype [$F_{(2,21)} = 6.16$, $P = 0.007$] but no L-AAA CM x spine subtype interaction [$F_{(2,21)} = 2.365$, $P = 0.11$]. A two-way ANOVA of spine density on basal dendrites showed an effect of L-AAA CM [$F_{(1,21)} = 15.37$, $P < 0.001$], an effect of the spine subtype [$F_{(2,21)} = 4.189$, $P = 0.029$] and a L-AAA CM x spine subtype interaction [$F_{(2,21)} = 4.223$, $P = 0.028$]. Post hoc comparisons revealed that neurons treated with L-AAA CM (0.05mM) had lower thin spine density on apical dendrites compared to control-treated neurons (CM) [$P < 0.001$] and lower mushroom spine density [$P < 0.05$] and thin spine density [$P < 0.001$] on basal dendrites when compared to control-treated neurons (CM) (**Fig 3.8.G,H**).

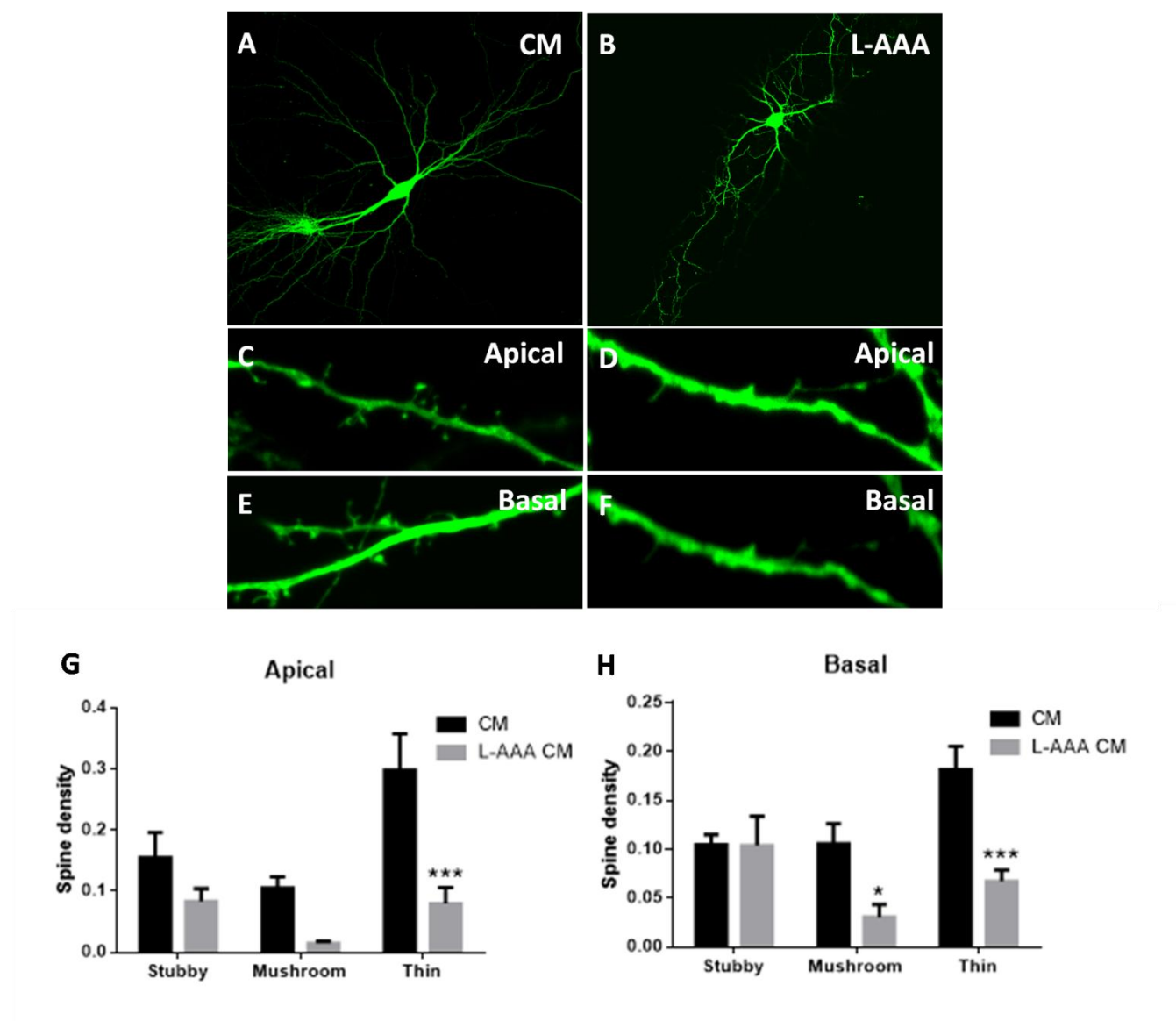


Figure 3.8: Effect of L-AAA-treated astrocytic CM on dendritic spine density in primary hippocampal neurons

Hippocampal neurons were transfected at DIV 7 with Lipofectamine 2000 (LF2000) and GFP DNA plasmid. Cells were treated at DIV15 for 24 h with L-AAA CM from astrocytes (0.05mM). Representative picture for CM (A,C,E) and representative picture for L-AAA CM (B,D,F). L-AAA CM affects thin spine density on apical dendrites (G) and mushroom and thin spine densities on basal dendrites (H). Data are expressed as mean $\pm$ SEM, (CM= 5 neurons; L-AAA= 4 neurons) representative of 1 independent experiment ***P<0.001 **P<0.01 and *P<0.05 vs. controls.

5.5. Effect of direct application of L-AAA on mRNA expression of astrocytic markers, OCR and ECAR after 24 h exposure

Purified astrocytes were treated for 24 h with L-AAA (0.5mM) or control cDMEM. Cells were harvested for RNA extraction followed by RT-PCR to analyse the expression of mRNA astrocytic markers. Student's T-test showed that L-AAA decreased GFAP expression [$T_{(10)}=10.11$, $P<0.001$] when compared to control cDMEM (**Fig 3.9.1A**). Student's T-test showed that L-AAA decreased GLAST expression [$T_{(10)}=10.59$, $P<0.001$] when compared to control NBM (**Fig 3.9.1B**). Student's T-test showed no effect of L-AAA on S100b expression [$T_{(10)}=0.79$, $P=0.44$] when compared to control cDMEM (**Fig 3.9.1C**). Student's T-test showed no effect of L-AAA on caspase 3 expression [$T_{(9)}=0.8895$, $P=0.396$] when compared to control cDMEM (**Fig 3.9.1D**).

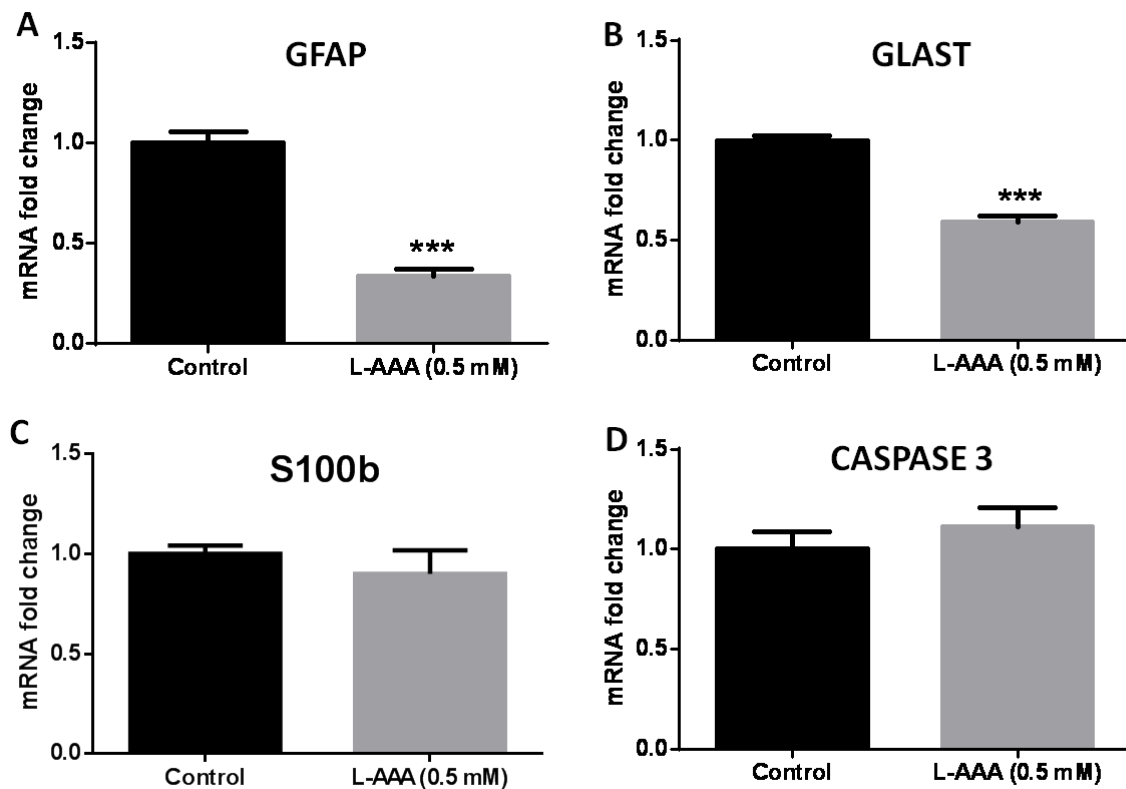


Figure 3.9.1: Effect of 24 h direct application of L-AAA on mRNA expression of astrocytic markers

Primary cortical astrocytes (DIV 13) were treated for 24 h with L-AAA (0.5mM) or control cDMEM. Cells were harvested for RNA extraction followed by RT-PCR to analyse the expression of **A)** GFAP, **B)** GLAST, **C)** S100b **D)** Caspase 3. Data are expressed as mean $\pm$ SEM, $n=6$ wells per group. *** $P<0.001$ ** $P<0.01$ and * $P<0.05$ vs. Control cDMEM.

Purified astrocytes were treated for 24 h with L-AAA (0.5mM) or control cDMEM. ECAR and OCR were measured using an Extracellular FluxXF-24 Analyzer (Seahorse Biosciences). There was no effect of L-AAA on basal glycolysis [$T_{(8)}=0.3753$, $P=0.717$] (**Fig 3.9.2.A**), glycolytic capacity [$T_{(8)}=0.3486$, $P=0.736$] or glycolytic reserve [$T_{(8)}=0.186$, $P=0.857$] (data not shown) when compared to control. L-AAA decreased basal mitochondrial respiration [$T_{(8)}=2.759$, $P=0.024$], maximal mitochondrial respiration [$T_{(8)}=5.228$, $P<0.001$] and ATP-linked mitochondrial respiration [$T_{(8)}=3.203$, $P=0.0126$] when compared to control.(**Fig 3.9.2-B-D**) [Student's t test].

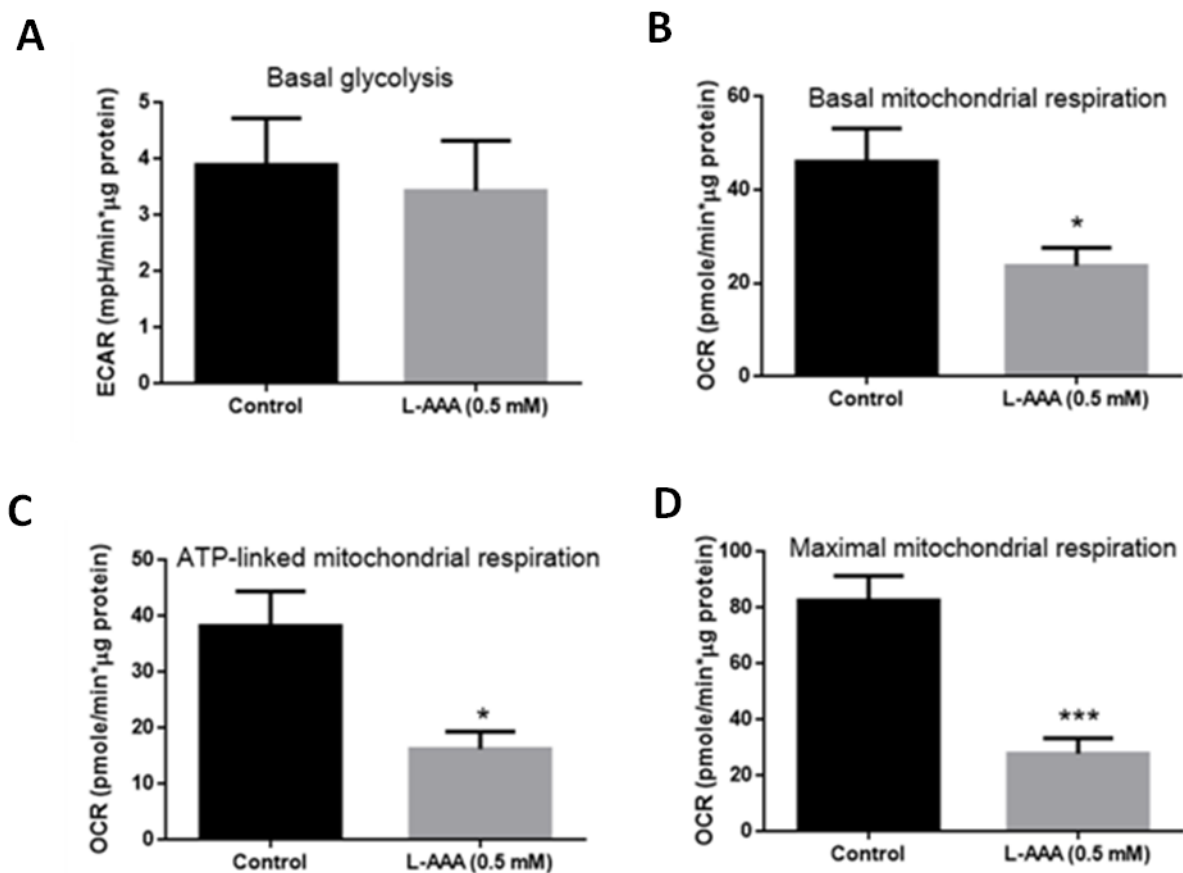


Figure 3.9.2: Effects of L-AAA on glycolysis and OCR in primary cortical astrocytes

Primary cortical astrocytes (DIV 13) were treated for 24 h with L-AAA (0.5mM) or control cDMEM and ECAR and OCR were measured using an Extracellular Flux Analyzer (Seahorse Biosciences). Basal glycolysis **A**), basal mitochondrial respiration **B**), ATP-linked mitochondrial respiration **C**), maximal mitochondrial respiration **D**). Data are expressed as mean $\pm$ SEM, $n=5$ replicates [3-5 wells per treatment per replicate (5)] *** $P<0.001$, ** $P<0.01$, * $P<0.05$ vs. Control cDMEM.

5.6. Effect of inhibitors of NMDA-R/NO signalling on astrocytic L-AAA CM-induced reduction in neurite outgrowth.

Considering their reported protective effects (Doucet et al., 2015) we investigated if ketamine, TRIM and inhibitors of PSD-95/nNOS interaction protect neurons from L-AAA CM-induced atrophy.

Two-way ANOVA of the number of neuritic branches showed an effect of L-AAA CM [$F_{(1,297)} = 20.62$, $P < 0.001$], an effect of the treatment [$F_{(4, 297)} = 6.56$, $P < 0.001$] but no L-AAA CM x treatment interaction [$F_{(4,297)} = 1.7$, $P = 0.15$]. Post hoc comparisons revealed that neurons treated with L-AAA CM had fewer neuritic branches when compared to control-treated neurons [$P < 0.001$]. Treatment with TRIM attenuated the L-AAA treated astrocyte CM-induced reduction in neuritic branches [$P < 0.001$]. Attenuation was not evident following treatment with ketamine, IC87201 or ZL006 (**Fig 3.10A**). Two-way ANOVA of neuritic length showed an effect of L-AAA CM [$F_{(1, 292)} = 10.97$, $P = 0.001$], an effect of treatment [$F_{(4,292)} = 11.38$, $P < 0.001$] and a L-AAA CM x treatment interaction [$F_{(4, 292)} = 2.67$, $P = 0.032$]. Post hoc comparisons revealed that neurons treated with L-AAA CM had shorter neuritic length when compared to control-treated neurons [$P < 0.001$]. TRIM increased the neuritic length of neurons not exposed to L-AAA treated astrocyte CM when compared to vehicle control [$P < 0.001$] and treatment with TRIM, IC87201 and ZL006 attenuated the L-AAA treated astrocyte CM-induced reduction in neuritic length [$P < 0.001$]. (**Fig 3.10B**).

Two-way ANOVA of the number of primary neurites showed no effect of L-AAA CM [$F_{(1,298)} = 1.41$, $P = 0.23$], an effect of treatment [$F_{(4, 298)} = 3.38$, $P = 0.01$] and a L-AAA CM x treatment interaction [$F_{(4,298)} = 5.85$, $P < 0.001$]. Post hoc comparisons revealed that neurons treated with L-AAA had fewer primary neurites when compared to control-treated neurons [$P < 0.001$]. Treatment with TRIM [$P < 0.01$] and ZL006 [$P < 0.001$] attenuated the L-AAA treated astrocyte CM-induced reduction in the number of primary neurites (**Fig 3.10C**).

In an independent experiment, the effect of a high dose of ketamine (300nM) on neuronal complexity was investigated (data not shown). Two-way ANOVA of the number of neuritic branches showed an effect of L-AAA CM [$F_{(1,57)} = 12.72$, $P < 0.001$], but failed to demonstrate an effect of ketamine [$F_{(1,57)} = 1.2$, $P = 0.27$] or an L-AAA CM x ketamine interaction [$F_{(1,57)} = 2.03$, $P = 0.15$]. A two-way ANOVA of the neuritic length showed an effect of L-AAA CM [$F_{(1,57)} = 6.79$, $P = 0.011$], no effect of ketamine [$F_{(1,57)} = 0.36$, $P = 0.54$] and no L-AAA CM x ketamine

interaction [$F_{(1,57)} = 2.66$, $P = 0.1$]. Finally, a two-way ANOVA of the number of primary neurites showed an effect of L-AAA CM [$F_{(1,57)} = 7.17$, $P = 0.0096$] no effect of ketamine [$F_{(1,57)} = 3.39$, $P = 0.07$] or L-AAA CM x ketamine interaction [$F_{(1,57)} = 3.81$, $P = 0.055$].

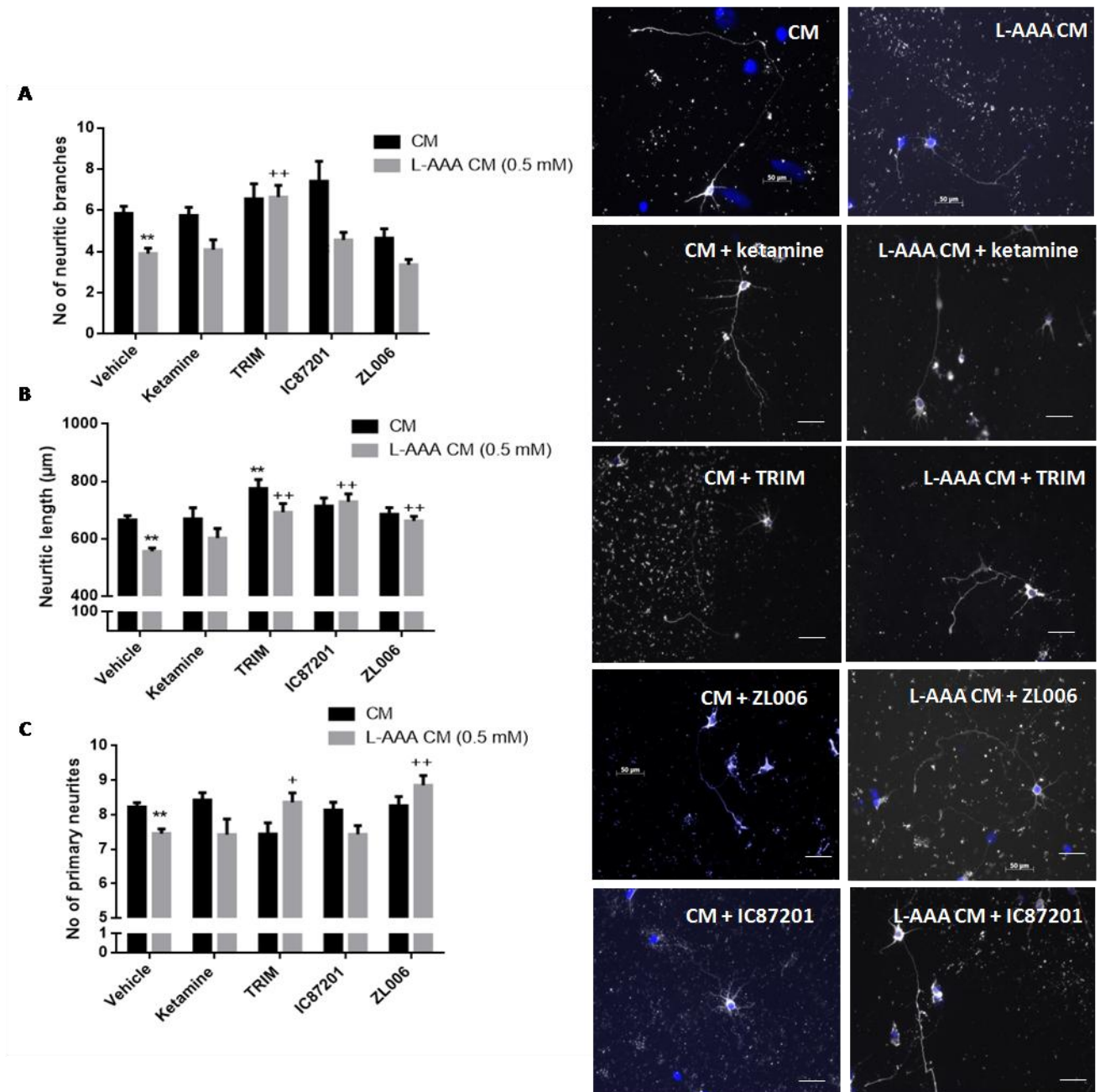


Figure 3.10 Effects of ketamine, TRIM, ZL006 and IC87201 on L-AAA treated astrocyte CM-induced reduction in neural complexity

Primary cortical neurons (DIV 3) were treated for 24 h with a combination of CM from healthy or L-AAA (0.5 mM) treated astrocytes and vehicle, ketamine (100 nM), TRIM (100 nM), ZL006 (100 nM) or IC87201 (100 nM). Sholl analysis was performed on neurons to examine A) the number of neuritic branches, B) neuritic length C) the number of primary branches. Data are expressed as mean $\pm$ SEM, $n = 16-24$ coverslips per group representative of 3 independent experiments for ketamine, TRIM, IC87201 and ZL006. Data for vehicle CM and L-AAA CM groups are pooled together from all experiments. ** $P < 0.001$, * $P < 0.01$ vs. control vehicle, ++ $P < 0.001$, + $P < 0.01$ vs. vehicle L-AAA CM.

5.7. Effect of inhibitors of NMDA-R/NO signalling on astrocytic L-AAA CM-induced reduction in synapse formation.

Since astrocyte CM increased neurite outgrowth, we assessed the effects of astrocyte CM on formation of synaptic puncta in primary cortical neurons (DIV 20) as assessed by the co-localisation of the pre- and post-synaptic markers, synaptophysin and PSD-95. CM from healthy astrocytes increased the number of synaptic puncta [$T_{(18)}=2.46$, $P=0.02$] when compared to control [Student's T test]. One way ANOVA of the synaptic puncta showed an effect of L-AAA (0.05 and 0.5 mM) treated astrocyte CM [$F_{(2,26)}=28.57$, $P<0.001$]. Post hoc comparisons revealed that L-AAA CM provoked a reduction in the number of synaptic puncta when compared to neurons treated with CM alone [$P<0.001$] (**Fig 11.A**).

There was no effect of CM from healthy astrocytes on PSD-95 [$T_{(18)}=0.36$, $P=0.71$] or synaptophysin [$T_{(18)}=0.84$, $P=0.41$] immunoreactivity when compared to control cNBM [Student's T test]. One way ANOVA of PSD-95 and synaptophysin immunoreactivity showed effects of L-AAA CM [$F_{(2,26)}=15.14$, $P<0.001$] and [$F_{(2,26)}=23.68$, $P<0.001$] respectively. Post hoc comparison revealed that L-AAA CM (0.05 mM and 0.5 mM) provoked a decrease in PSD-95 and synaptophysin immunoreactivity when compared to neurons treated with CM alone [$P<0.001$] (**Fig 11 B-C**).

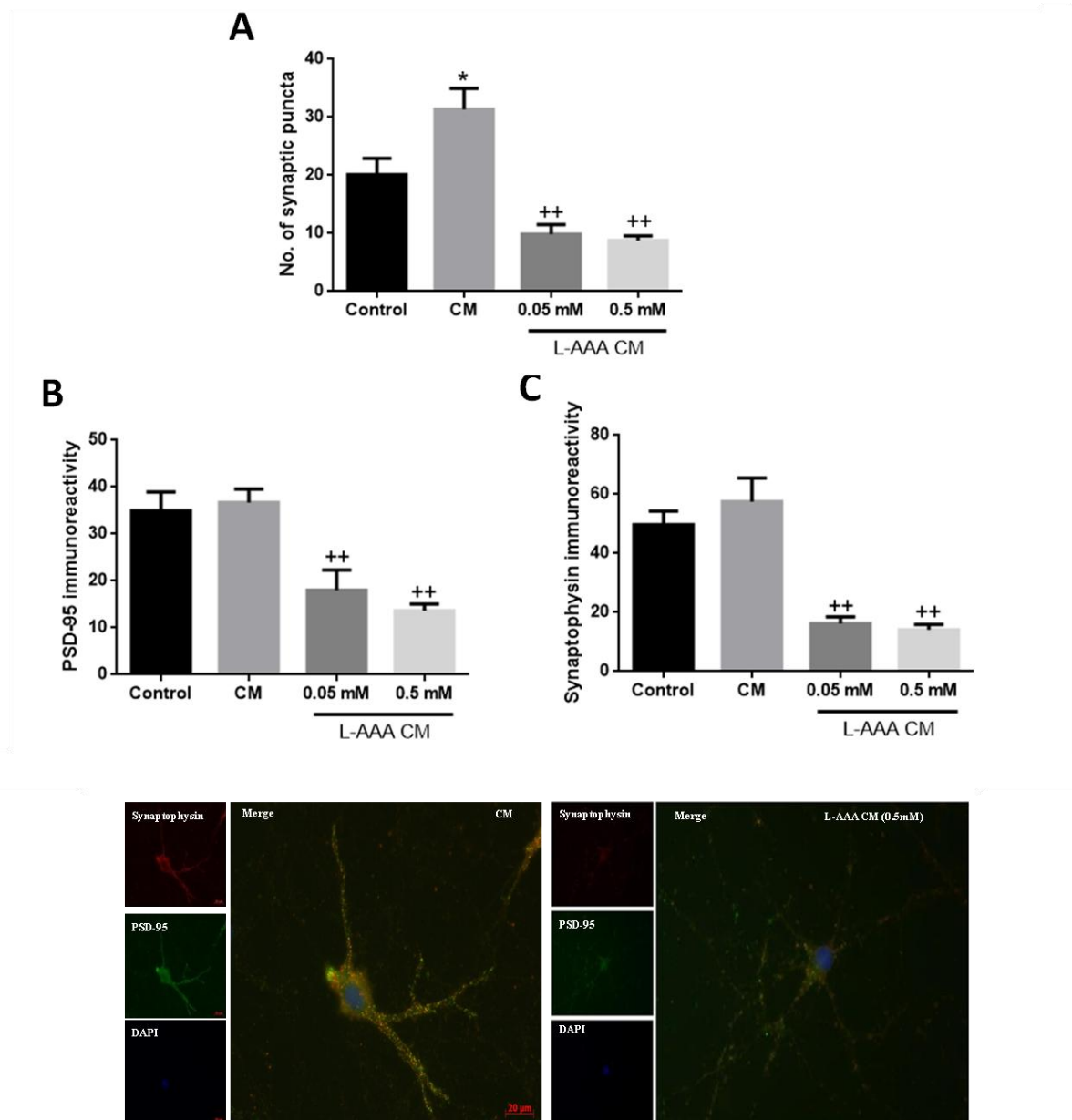


Figure 3.11: Effects of astrocyte CM on the co-localised expression of synaptophysin and PSD-95 in synaptic puncta

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. Quantification of co-localised synaptic proteins was conducted using ImageJ software. A) Co-localised puncta from neurons were treated for 24 h with normal culture media (control), 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). B) PSD-95 immunoreactivity or C) Synaptophysin immunoreactivity. Data are expressed as mean $\pm$ SEM, $n=9-10$ coverslips per group ** $P<0.01$, *** $P<0.001$ vs. control NBM, +++ $P<0.001$ vs. control CM.

Since astrocyte L-AAA CM reduced synapse formation in primary cortical neurons (DIV 20), we next investigated if inhibitors of NMDA-R/NO signalling could rescue the reductions in the number of co-localised puncta following L-AAA CM treatment. For ketamine treatment, two-way ANOVA of the number of synaptic puncta demonstrated effects of L-AAA CM [$F_{(1,79)}=9.56$, $P=0.0027$], no effect of ketamine [$F_{(1,79)}=1.8$, $P=0.18$] but a L-AAA CM x

ketamine interaction [$F_{(1,79)} = 6.76$, $P = 0.011$]. Post hoc comparisons revealed a decrease in the number of synaptic puncta following L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with ketamine attenuated the L-AAA CM-induced reduction in number of synaptic puncta [$P < 0.01$] (**Fig 3.12A**). Two-way ANOVA of PSD-95 immunoreactivity showed effects of L-AAA CM [$F_{(1,79)} = 13.61$, $P = 0.0004$], no effect of ketamine [$F_{(1,79)} = 0.63$, $P = 0.428$] and no L-AAA CM x ketamine interaction [$F_{(1,79)} = 3.589$, $P = 0.0618$]. Post hoc comparisons revealed a decrease in PSD-95 immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$] (**Fig 3.12B**). Two-way ANOVA of synaptophysin immunoreactivity showed effects of L-AAA CM [$F_{(1,79)} = 13.45$, $P < 0.001$], an effect of ketamine [$F_{(1,79)} = 7.49$, $P = 0.0077$] and a L-AAA CM x ketamine interaction [$F_{(1,79)} = 12.29$, $P < 0.001$]. Post hoc comparisons demonstrated a decrease in synaptophysin immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with ketamine attenuated the L-AAA CM-induced reduction in synaptophysin immunoreactivity [$P < 0.001$] (**Fig 3.12C**).

We also assessed the effect of TRIM on synaptic puncta. Two-way ANOVA of the number of synaptic puncta showed effects of L-AAA CM [$F_{(1,82)} = 21.44$, $P < 0.001$], an effect of TRIM [$F_{(1,82)} = 5.53$, $P = 0.021$] and no L-AAA CM x TRIM interaction [$F_{(1,82)} = 1.899$, $P = 0.17$]. Post hoc comparisons revealed a decrease in the number of synaptic puncta following L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with TRIM attenuated the L-AAA CM-induced reduction in the number of synaptic puncta [$P < 0.01$] (**Fig 3.12D**). Two-way ANOVA of PSD-95 immunoreactivity showed effects of L-AAA CM [$F_{(1,82)} = 17.12$, $P < 0.001$], no effect of TRIM [$F_{(1,82)} = 0.067$, $P = 0.79$], but a L-AAA CM x TRIM interaction [$F_{(1,82)} = 5.1$, $P = 0.026$]. Post hoc comparisons revealed a decrease in PSD-95 immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$] (**Fig 3.12E**). Two-way ANOVA of synaptophysin immunoreactivity showed effects of L-AAA CM [$F_{(1,82)} = 27.18$, $P < 0.001$], an effect of TRIM [$F_{(1,82)} = 10.83$, $P = 0.0015$] and a L-AAA CM x TRIM interaction [$F_{(1,82)} = 4.055$, $P = 0.047$]. Post hoc comparisons revealed a decrease in synaptophysin immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with TRIM attenuated the L-AAA CM-induced reduction in synaptophysin immunoreactivity [$P < 0.001$] (**Fig 3.12F**).

Since NMDA-R modifiers were able to rescue the synapse loss observed after L-AAA CM treatment, we were interested to see if PSD-95/nNOS modifiers could rescue the reduction in synaptic puncta observed following L-AAA CM treatment. Two-way ANOVA of the number of synaptic puncta showed effects of L-AAA CM [$F_{(1,83)} = 9.98$, $P = 0.002$], no effect of IC87201 [$F_{(1,83)} = 0.03$, $P = 0.86$] but a L-AAA CM x IC87201 interaction [$F_{(1,83)} = 9.14$, $P = 0.003$]. Post hoc comparisons revealed a decrease in the number of synaptic puncta following L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$] (**Fig 3.13A**).

Two-way ANOVA of PSD-95 immunoreactivity showed effects of L-AAA CM [$F_{(1,83)} = 10.77$, $P = 0.0015$], no effect of IC87201 [$F_{(1,83)} = 0.044$, $P = 0.83$] and a L-AAA CM x IC87201 interaction [$F_{(1,83)} = 9.72$, $P = 0.0025$]. Post hoc comparisons revealed a decrease in PSD-95 immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$] (**Fig 3.13B**). Two-way ANOVA of synaptophysin immunoreactivity revealed effects of L-AAA CM [$F_{(1,83)} = 14.17$, $P < 0.001$], no effect of IC87201 [$F_{(1,83)} = 0.79$, $P = 0.39$], but a L-AAA CM x IC87201 interaction [$F_{(1,83)} = 15.56$, $P < 0.001$]. Post hoc comparisons revealed a decrease in synaptophysin immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with IC87201 attenuated the L-AAA CM-induced reduction in synaptophysin immunoreactivity [$P < 0.01$] (**Fig 3.13C**).

We also assessed the effect of ZL006 on the number of synaptic puncta. Two-way ANOVA of the number of synaptic puncta showed effects of L-AAA CM [$F_{(1,97)} = 9.72$, $P = 0.002$], no effect of ZL006 [$F_{(1,97)} = 2.43$, $P = 0.12$] but a L-AAA CM x ZL006 interaction [$F_{(1,97)} = 9.3$, $P = 0.003$]. Post hoc comparisons revealed a decrease in the number of synaptic puncta following L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with ZL006 attenuated the L-AAA CM-induced reduction in synaptic puncta [$P < 0.01$] (**Fig 3.13D**). Two-way ANOVA of PSD-95 immunoreactivity showed effects of L-AAA CM [$F_{(1,96)} = 6.72$, $P = 0.01$], an effect of ZL006 [$F_{(1,96)} = 7.907$, $P = 0.006$], and a L-AAA CM x ZL006 interaction [$F_{(1,96)} = 10.18$, $P = 0.0019$]. Post hoc comparisons revealed a decrease in PSD-95 immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P < 0.001$]. Treatment with ZL006 attenuated the L-AAA CM-induced reduction in PSD-95 immunoreactivity [$P < 0.001$] (**Fig 3.13E**). Two-way ANOVA of synaptophysin immunoreactivity showed effects of L-AAA CM [$F_{(1,96)} = 10.49$,

P=0.0017], no effect of ZL006 [$F_{(1,96)} = 3.310$, $P=0.07$], but a L-AAA CM x ZL006 interaction [$F_{(1,96)} = 12.10$, $P<0.001$]. Post hoc comparisons revealed a decrease in synaptophysin immunoreactivity after L-AAA CM treatment when compared to neurons treated with healthy CM alone [$P<0.001$]. Treatment with ZL006 attenuated the L-AAA CM-induced reduction in synaptophysin immunoreactivity [$P<0.001$] (**Fig 3.13F**).

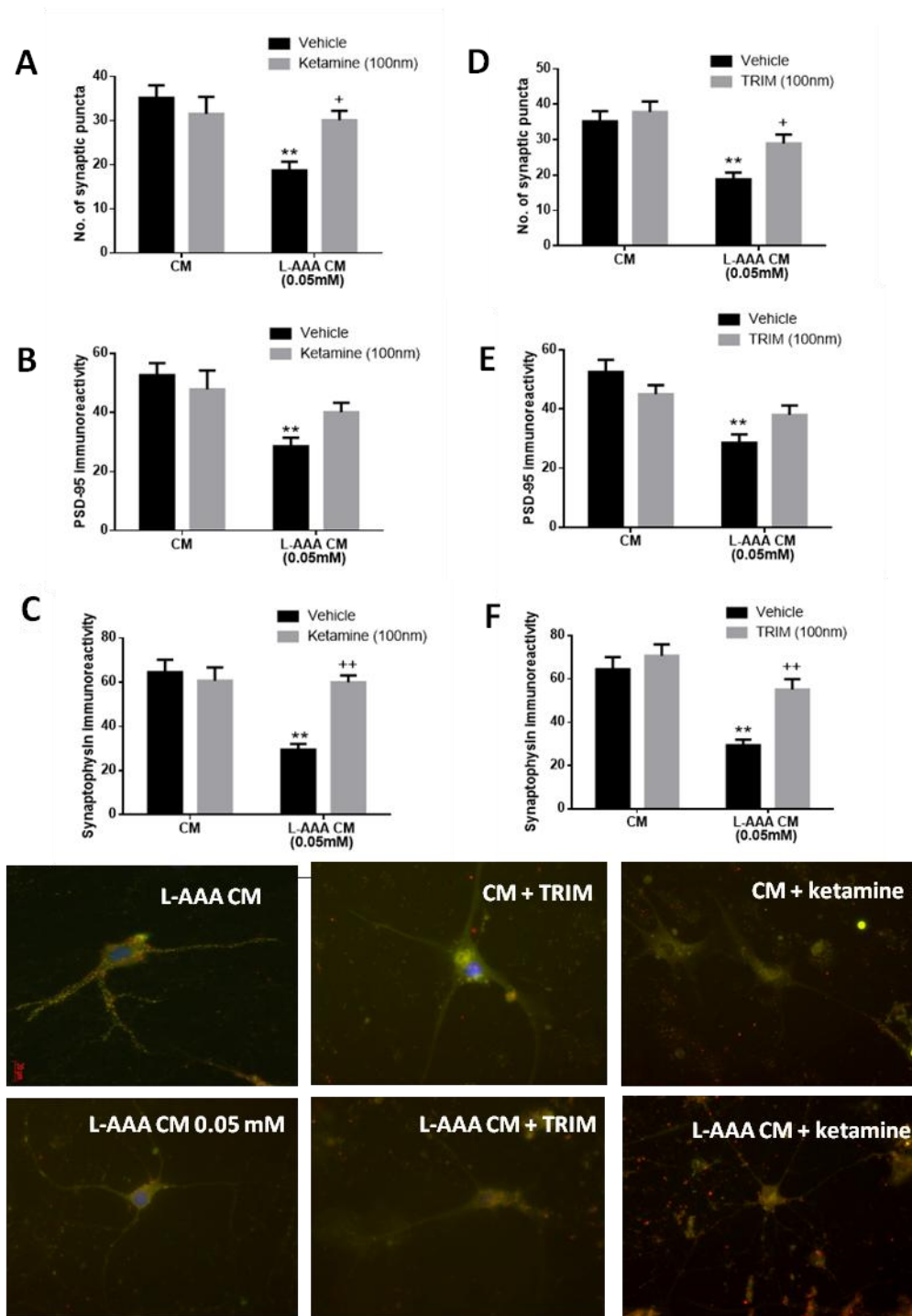


Figure 3.12. Effects of ketamine and TRIM on the co-localised expression of synaptophysin and PSD-95 in synaptic puncta.

Synaptic puncta were quantified on neurons treated for 24 h with a combination of L-AAA (0.05mM) or control CM in addition to ketamine; synaptic puncta **A**, PSD-95 immunoreactivity **B**, synaptophysin immunoreactivity **C**. In addition to TRIM; synaptic puncta **D**, PSD-95 immunoreactivity **E**, synaptophysin immunoreactivity **F**). (n=13-24 coverslips representative of 3 independent experiments, data for vehicle CM and L-AAA CM groups are pooled together from all experiments, n=27-30 coverslips per group representative of 4 independent experiments. Data are expressed as mean $\pm$ SEM. Post hoc comparisons *P<0.01, **P<0.001 vs. control CM, +P<0.01, ++P<0.001 vs. respective vehicle L-AAA CM.

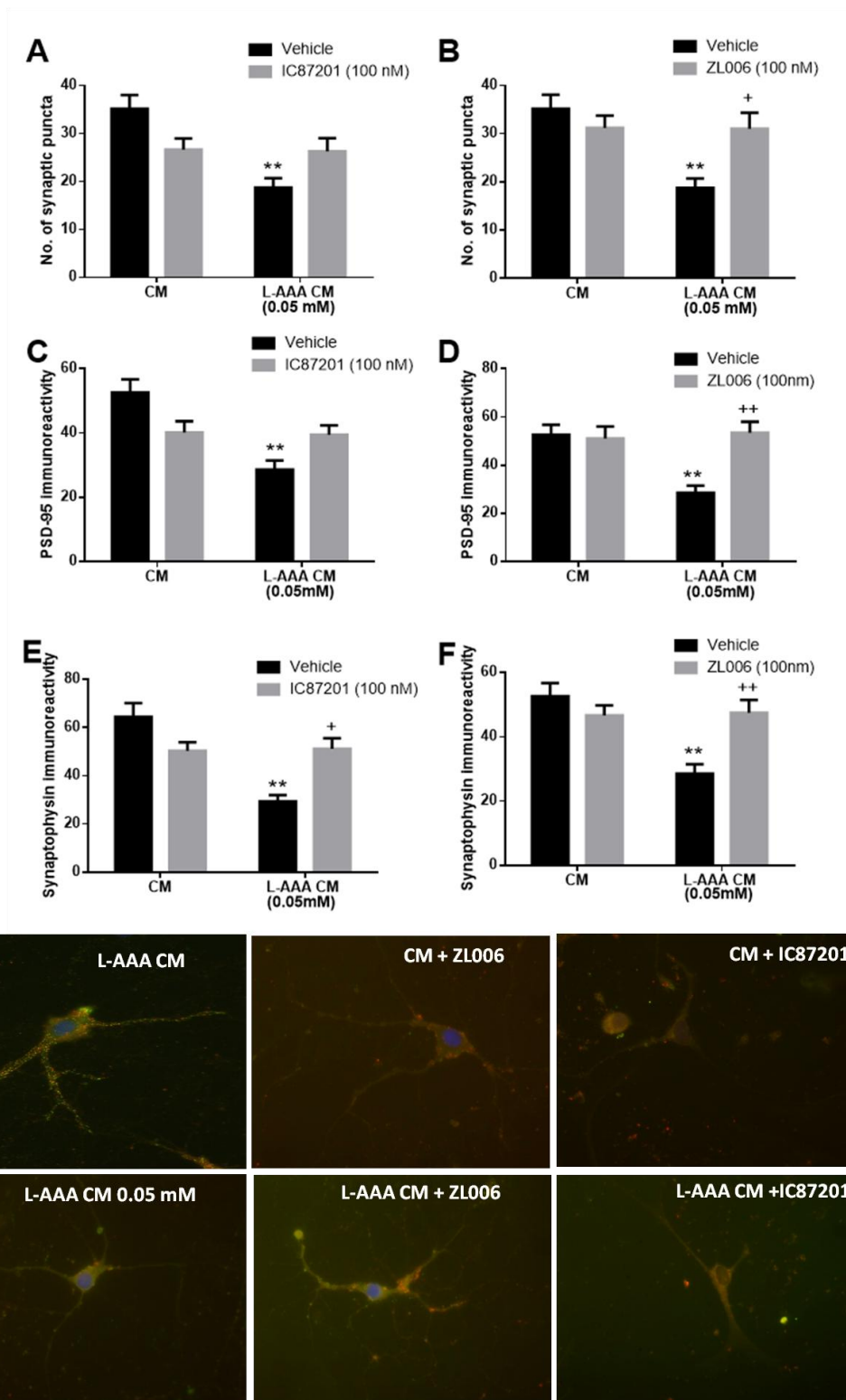


Figure 3.12. Effects of IC87201 and ZL006 on the co-localised expression of synaptophysin and PSD-95 in synaptic puncta.

Synaptic puncta were quantified on neurons treated for 24 h with a combination of L-AAA (0.05mM) or control CM in addition to IC87201; synaptic puncta **A**, PSD-95 immunoreactivity **B**, synaptophysin immunoreactivity **C**). In addition to ZL006; synaptic puncta **D**, PSD-95 immunoreactivity **E**, synaptophysin immunoreactivity **F**). (n=13-24 coverslips representative of 3 independent experiments, data for vehicle CM and L-AAA CM groups are pooled together from all experiments, n=27-30 coverslips per group representative of 4 independent experiments. Data are expressed as mean $\pm$ SEM. Post hoc comparisons *P<0.01, **P<0.001 vs. control CM, +P<0.01, ++P<0.001 vs. respective vehicle L-AAA CM.

6. Discussion

Results demonstrate that CM derived from healthy astrocytes increases neurite outgrowth in immature neurons and synapse number in mature neurons. Direct application of L-AAA to astrocytes provoked a reduction in the astrocytic markers GFAP and GLAST. L-AAA resulted in altered mitochondrial respiration without affecting glycolysis. On the other hand, CM derived from L-AAA-treated astrocytes impaired neurite outgrowth in immature primary cortical neurons and reduced synapse formation in mature neurons. Lastly, this study demonstrated that treatment with NMDA-R/NO signalling inhibitors can attenuate both neuronal atrophy and synapse loss induced by astrocytic L-AAA CM. The nNOS inhibitor TRIM and small molecule inhibitors of the PSD-95/nNOS interaction, IC87201 and ZL006 induced a partial protective effect from L-AAA CM-induced effects on neurite outgrowth whereas ketamine, TRIM, ZL006 and IC87201 induced a partial protective effect from L-AAA CM-induced effects on synapse number.

6.1. Effect of astrocytes CM on neurite outgrowth and synapse formation

Results of the present study demonstrate that CM derived from healthy astrocytes increases neurite outgrowth and synapse formation in primary cortical neurons. Specifically, we showed that CM harvested from healthy astrocytes at 24 h and transferred to primary cortical neurons for 24 h results in an increase in primary neurites and neuritic branches. These results demonstrate that untreated (healthy) astrocytes produce a trophic media which can increase neurite outgrowth when transferred onto primary cortical neurons *in vitro*. These findings are in line with literature indicating a neurotrophic effect of astrocyte CM. Pre-treatment with untreated astrocyte CM has been shown to attenuate glutamate-induced apoptotic cell death in rat spinal cord neurons (Lu et al., 2015). Astrocyte CM has also been shown to produce neuroprotective effects against the environmental chemical methylmercury via BDNF and NGF release (Takemoto et al., 2015), to protect primary cultures of hippocampal neurons from corticosterone induced damage (Zhu et al., 2006), as well as partially attenuating neurotoxic effects associated with excessive ROS and reactive nitrogen species (RNS) (Tanaka et al., 1999). A recent study found that astrocytes attenuated the neurotoxic effects induced by pro-inflammatory cytokines, tumor necrosis factor (TNF) and interleukin 1 (IL-1) in a fetal human mesencephalic cell line (LUHMES) and immortalized murine astrocytes (IMA) (Efremova et al., 2017). These results confirm the role of astrocytes in

neuronal outgrowth and synapse formation and support the use of astrocyte CM as a method of enhancing neurotrophic support and neuroprotection against harmful stimulants.

24 h treatment of primary mature cortical neurons (DIV 20) with astrocyte CM has been shown to increase synapse formation. Our results are line with previous research demonstrating increased co-localised synaptophysin and PSD-95 immunoreactivity with no change in synaptophysin and PSD-95 immunoreactivity and combined these results, suggest that astrocyte CM increases synapse formation *in vitro* (Pfrieger and Barres, 1997; Ippolito and Eroglu, 2010; O'Neill et al., 2016). The mechanism by which astrocytes promote synapse formation has been extensively reviewed in the literature and shown to involve TSP-1 in the fragile X mouse model (Cheng et al., 2016) and Growth Factor Beta 1 to protect synapses against A β Oligomers *in vitro* (Pereira Diniz et al., 2017).

6.2. Effect of direct application of L-AAA on viability of neurons and astrocytes

Direct application of L-AAA (0.5mM) for 48 h had no effect either astrocyte or neuronal viability. These results differ from the literature which demonstrate reduced astrocytic viability following application of higher concentrations of L-AAA (1–10mM) when analysed by the MTS assay (Brown and Kretschma, 1998). These differences could be attributed to the difference in L-AAA concentration (0.5mM vs. 1-10mM), the level of maturation of cells at the time of treatment (DIV 13 vs. day of seeding), the treatment duration (48 h vs. 5 days) and the type of astrocytes under investigation (mixed vs. type 1/ type 2) (Brown and Kretschma, 1998). Although early research suggest that L-AAA can exhibit excitotoxic effects on neurons at high concentrations *in vitro* (MacDonald and Wojtowicz, 1982), in this present investigation, L-AAA did not affect the viability of neuronal cultures. It was of importance to establish that L-AAA did not affect neuronal viability in our model since we were interested in examining the impact of astrocytic dysfunction, astrocyte derived secreted factors or lack of astrocytes secreted factors on neuronal responses, rather than any direct effect of L-AAA.

6.3. Effect of direct application of L-AAA on mRNA astrocytic markers OCR and ECAR

This research further investigated the mechanism of action of L-AAA by quantifying mRNA expression of astroglial specific markers. L-AAA treatment applied for 24 h on primary cortical astrocytes (0.5mM) decreased mRNA expression levels of the prototypical astrocyte

marker, GFAP. These results which demonstrate a decrease in GFAP expression, suggest that L-AAA does not cause the classical ‘reactive astrogliosis’ response in astrocytes (Sofroniew, 2009; Zamanian et al., 2012) but instead leads to an impairment associated with reduced expression of this marker. 24 h treatment with L-AAA (0.5mM) had no effects on S100b mRNA expression levels; however it resulted in a decrease expression of GLAST mRNA in astrocytes. Alterations in mRNA expression of GLAST suggest that the astrocytic glutamate transport may be affected by this toxin. Given that astrocytes play an important role in glutamate regulation, these results correspond with the literature reinstating that L-AAA by targeting astrocytes induces alterations in the astrocytic glutamate transport systems (McBean, 1994; Tsai et al., 1996; Brown and Kretzschma, 1998). However, measuring GFAP and GLAST at mRNA level in primary astrocytes following L-AAA exposure is quite limited. To complete this analysis, it would have been interesting to perform immunohistochemistry against those markers in fixed cells.

Previous research has demonstrated that astrocytes die following prolonged exposure to L-AAA (1mM), suggesting activation of delayed apoptotic mechanisms (Nishimura et al., 2000). This research shows that L-AAA (0.5mM) has no effect on caspase-3 mRNA expression after 24 h of treatment, suggesting that L-AAA-dependent cell toxicity at this dose is not linked to caspase 3 mRNA expression. Although caspase activity or Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) staining would provide better measure of apoptosis as caspase-3 activation is reported as a late event in the apoptotic cascade, caspase-3 mRNA up-regulation precedes caspase-3 enzyme activation in cells undergoing apoptosis. Indeed, caspase-3 mRNA expression occurs 6h following induction of ischemia indicating apoptosis initiation at this early stage of ischemia-induced cell death (Harrison et al., 2000).

This study also demonstrated that astrocytes treated with L-AAA (0.5mM) for 24 h exhibit a reduction in OCR, an indicator of mitochondrial respiration (Ferrick et al., 2008) but had no effect on glycolysis. In particular, basal OCR, maximal OCR and ATP-linked mitochondrial respiration were impaired following L-AAA treatment. This decrease of mitochondrial respiration may be due to an accumulation of glutamate in astrocytes as previously mentioned following glutamate treatment (Yan et al., 2017). In addition, glutamine, along with glucose is essential for the function of the Krebs’s Cycle which produces ATP within the cell for energy requirements (please refer to **Fig 2.8** in chapter 2) (Yang et al., 2014). ATP is also needed in many enzymatic reactions within cells. GS, the enzyme that converts glutamate

into glutamine requires ATP to carry out this process. In astrocytes, the sodium-potassium ATPase and glutamate transporters are tightly coupled (Parpura et al., 2012). Previous research demonstrated that ATP depletion affects glutamate transport as glutamate uptake requires ATP produced from mitochondrial respiration (Madl and Burgesser, 1993). Interestingly, in astrocytic culture *in vitro* and in brain *in vivo*, mitochondrial respiration impairment or oxygen deprivation provoked a reduction in glutamate transporter capacity (Longuemare et al., 1994; Swanson et al., 1994; Ouyang et al., 2007).

6.4. Effect of CM from L-AAA-treated astrocytes on neurite outgrowth, synapse formation and dendritic spine density

L-AAA CM (0.5mM) applied for 24 h onto neurons reduced neuritic length and the number of primary neurites and neuritic branches of neurons compared to neurons treated with CM only. The effects of L-AAA CM are similar to those of NMDA/glycine-induced neuronal atrophy observed by Doucet et al. (2015). These results demonstrated that, although L-AAA CM does not result in a reduction of astrocytic viability after 48 h, the CM from these cells produced a significant decrease in neurite length and branching after 24 h of treatment. L-AAA attenuated the trophic effects of astrocyte CM on neurite outgrowth. Importantly, direct application of L-AAA onto neurons did not affect neurite outgrowth after 48 h treatment. Instead L-AAA, by impairing astrocyte function, indirectly reduced their ability to promote neurite outgrowth. Taking into account the toxic effect of glutamate excess, one possible mechanism of the action of L-AAA may involve glutamate transport impairment with impaired glutamate metabolism and clearance which may impact on neuronal outgrowth.

Furthermore, this investigation indicates that dysfunctional L-AAA-treated astrocytes (0.05 and 0.5mM) abolished the synaptogenic effects of astrocyte CM. Such observations are in line with a previous study demonstrating that cultured neurons exposed to CM from dysfunctional ammonia-treated astrocytes decreased the number of colocalised puncta (synaptophysin and PSD-95) (Jayakumar et al., 2014). Soluble factors secreted from dysfunctional astrocytes or lack of soluble neuroprotective factors from healthy astrocytes may negatively affect synapse formation and development as proposed in a range of CNS disorders including schizophrenia (Harrison, 2004), MDD (Cotter et al., 2001; Rajkowska and Miguel-Hidalgo, 2007) and Alzheimer's disease (Shankar et al., 2007).

One *in vitro* pilot study on mature hippocampal neurons transfected with GFP demonstrated that 24 h treatment of L-AAA-treated astrocytes CM (0.05mM) to neurons decreased thin

spine density on apical and basal dendrites and mushroom spine density on basal dendrites. After 24 h L-AAA CM decreased both immature (thin) and mature (mushroom) spines. Given the role of spines in synapse formation, those results are in accordance with the decrease in synapse number observed in cortical neurons after L-AAA CM treatment. Therefore, L-AAA-treated astrocyte CM not only decreases neuronal complexity but also affect spine dynamics and the formation of synapses.

6.5. Effect of NMDA-R/NO signalling inhibitors on astrocytic L-AAA CM-induced reduction in neurite outgrowth and synaptogenesis.

Inhibitors of the NMDA-R/NO signalling (100nM) were able to partially reverse L-AAA CM (0.5mM)-induced neuronal atrophy. However, the effects were mixed and differed for neuritic branches, neuritic length or the number of primary neurites. TRIM was the only drug able to reverse L-AAA CM-induced neuritic branches atrophy whereas TRIM, ZL006 and IC87201 were able to reverse L-AAA CM-induced reduction in neuritic length. Lastly, TRIM and ZL006 were able to reverse L-AAA CM-induced reductions in the number of primary neurites. Ketamine (100 or 300nM) didn't reverse any of the effect of L-AAA on neuronal complexity.

This research extended previous *in vitro* investigation from our lab (M.V. Doucet et al., 2015) showing that inhibitors of the NMDA-R/NO signalling pathway were able to counteract NMDA/glycine-induced neuronal atrophy. Although a similar partial rescue from neuronal atrophy was observed, the effect of the drugs on the parameters of neuronal complexity differed. Ketamine (10nM) reversed NMDA/glycine-induced reduction in primary neurites, neuritic branches and neuritic length but had no effect on L-AAA CM-induced neuronal atrophy at 100 or 300nM in this research. Although we expected to see a rescue from ketamine following L-AAA-induced neuronal atrophy, the absence of effect observed in this investigation may be due to the higher concentration used or to the use of L-AAA versus NMDA/gly. TRIM (100nM) reversed NMDA/glycine-induced reduction in neuritic branches and length and also counteracted L-AAA-induced reductions in the number of primary neurites in this investigation. TRIM by itself decreases the number of primary neurites. However, this might be associated to an increase in the neuritic length following TRIM treatment alone. IC87201 (100nM) previously reversed NMDA/glycine-induced reduction in primary neurites, neuritic branches and neuritic length but only reversed L-AAA CM-induced reduction in neuritic length in this investigation. Although it seems that IC87201 decreases

the number of synaptic puncta, this difference isn't significant and is associated with variability between the replicates. ZL006 (10 and 100nM) reversed NMDA/glycine-induced reduction in neuritic branches and length previously and in this investigation ZL006 reversed L-AAA CM-induced reduction in primary neurites and length.

These results suggest that NMDA-R antagonists, nNOS inhibitors and inhibitors of the PSD-95/nNOS interaction have differing effects on modulating L-AAA-induced and NMDA/glycine-induced neuronal atrophy. However, it confirms in two different models their ability to reverse the impact of direct and potential glutamate disturbance. Indeed, further research has demonstrated that ZL006 previously reversed the neuronal atrophy of cultured cortical neurons induced by high concentrations of glutamate (Zhou et al., 2010). One of the limitations of the current study includes that the Sholl analysis *in vitro* can only be performed on immature neurons to be able to visualise individual neurons. Although Doucet et al. (2015) demonstrated that immature neurons used in this experiment express PSD-95 and nNOS which are co-localised, more mature neurons have higher sensitivity to glutamate than immature neurons (Monnerie et al., 2003).

As synapses begin to form in cultured neurons only after 15 DIV (Dotti et al., 1988; Polleux and Snider, 2010), the effect of inhibitors of NMDA-R/NO signalling and L-AAA CM were assessed on mature neurons (DIV20). Treatment of neurons with ketamine, ZL006 or TRIM blocked L-AAA CM (0.05 mM)-induced reductions in the number of synaptic puncta formed in primary neuronal cultures. IC87201 showed a trend towards blockade of L-AAA CM (0.05mM)-induced reduction in the number of synaptic puncta. In each case the drug related attenuation of L-AAA CM induced loss was accompanied by a recovery of both synaptophysin and PSD-95 immunoreactivity. These results demonstrated that modulation of the NMDA-R/PSD-95/nNOS signalling pathway in cortical neurons can protect against atrophy and synapse loss associated with L-AAA-treated astrocyte CM.

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 and glutamate disturbance. In this regard, L-AAA has received particular attention in recent years as an effective method of inducing astrocyte ablation and depressive-like behaviours in rodents (Banasr and Duman, 2008; Lee et al., 2013; Liu F., et al., 2017). A recent study demonstrated that a single high dose of L-AAA (20µg/µl, 5µl, 1 injection) into the PFC of rats caused an astrocytic ablation

accompanied by progressive neuronal loss and a decrease of neuronal complexity of surviving neurons in the area of injection (Lima et al., 2014).

6.6. Conclusions

This work has extended on previous results from Doucet et al. (2015). Inhibitors of NMDA-R/NO signalling were able to partially reverse L-AAA CM-induced neuronal atrophy and synapse loss. However, some of the effects were partial or differed between the drugs.

A further limitation is that neurons used for Sholl analysis were immature. Therefore, transfecting mature neurons with GFP plasmids would allow for measurement of neuronal complexity to overcome this. A limitation of the methodological approach of this investigation is the analysis of spine density at just one point in time. Spine synapse formation is dynamic with a high turnover rate, therefore using time lapse two photon imaging would allow to measure spine changes over time. This research *in vitro* only focused on neuronal complexity, spine density and synapse formation. Neurite outgrowth in a neuronal network should be investigated. In this regard, live cell Ca^{2+} imaging has recently been used to examine neuronal network activity *in vitro* (Verstraelen *et al.*, 2014). In addition, it will be necessary to quantify glutamate uptake from astrocytes treated with L-AAA to determine its effect on glutamate metabolism in L-AAA-treated astrocytes and the implications of this for L-AAA-treated astrocytes CM-induced effects on neurons.

Testing inhibitors of the NMDA-R/NO signalling following L-AAA-induced astrocytic dysfunction in mice would serve to scale this work by assessing the effect of astrocytic dysfunction and inhibition of the NMDA-R/NO signalling on neuronal complexity, spine density and synapse formation *in vivo*. In addition, it would be of interest to assess the effect of L-AAA and inhibitors of the NMDA-R/NO signalling on behaviours as previous studies from our laboratory have reported these compounds to possess antidepressant-related properties in mice and rats (Doucet et al., 2013; Sherwin et al., 2017b) and that L-AAA injection into the PLC promotes depressive-related behaviour (Banasr and Duman, 2008).

Chapter 4

Delivery of L-AAA into the pre-limbic cortex (PLC) reduces GFAP and S100b immunoreactivity and provokes depressive-like behaviours in mice

1. Introduction

Astrocytes actively modulate synaptic transmission through their formation of a tripartite synapse, whereby one astrocyte is located in close proximity to a neuronal synapse (Haydon, 2001). Astrocytes are functionally linked to neurons via astrocytic end-feet: such that one single astrocyte in the human cortex may regulate the function of a number of neurons (Koehler et al., 2009). A diverse range of receptors and transporters for all the major neurotransmitter systems (glutamate, GABA, adrenaline, serotonin, dopamine, acetylcholine, and purine) are present in astrocytes which allow them to exert a homeostatic function in the brain. Astrocytes also play a role in the regulation of the inflammatory response system in the brain through the release of pro- and anti-inflammatory mediators (Chen et al., 2006; Rajkowska and Miguel-Hidalgo, 2007). Because of their involvement in numerous key mechanisms in the brain, astrocytes play a role in the underlying pathophysiology of a number of CNS disorders (De Keyser et al., 2008).

A number of post-mortem studies on MDD patients have shown that there are significant reductions in glial cell density in the fronto-limbic areas, especially in the PFC and medial PFC, the dorsolateral and orbito-frontal cortex (Rajkowska and Miguel-Hidalgo, 2007), subgenual cingulate cortex (Williams et al., 2012), amygdala (Bowley et al., 2002) and hippocampus (Stockmeier et al., 2004). Since astrocytes play a role in the removal of glutamate from the synapse, it has been proposed that an astrocytic deficit may account for alterations in glutamate neurotransmission in depression (Kugaya and Sanacora, 2005; Banasr and Duman, 2008; Niciu et al., 2012). Concentrations of glutamate and glutamine are reported to be higher in the plasma of depressed patients compared to healthy subjects (Küçükibrahimoğlu et al., 2009). In addition, MRS studies have shown alterations in glutamate and glutamine signals within different brain regions (Yüksel and Öngür, 2010) with an increase of glutamate/glutamine in the occipital cortex (Michael et al., 2003a) and a decrease in glutamate/glutamine levels in the PFC (Yalcin et al., 2005; Hasler et al., 2007;), anterior cingulate cortex (Auer et al., 2000; Rosenberg et al., 2005) and left amygdala (Michael et al., 2003a) reported in depressed patients when compared to healthy controls.

A promising animal model of astrocytic ablation or reduced astrocytic function has recently emerged which involves injecting L-AAA, an astrocyte specific toxin acting as a glutamate analogue which is taken up in a similar manner to glutamate via the sodium-dependent EAAT into the brain (Huck et al., 1984). Once taken up by the astrocytes, L-AAA competitively

inhibits GS and γ -glutamylcysteine synthase, contributing to dysregulated glutamate cycling (Olney et al., 1980; McBean, 1994).

One study reported on the effect of stereotactically delivered L-AAA (100 μ g/ μ l, 0.5 μ l, 2 injections) into the PLC of rats on depressive-like behaviours (Banasr and Duman, 2008). The study showed that 2 consecutive daily doses of L-AAA in the PLC was associated with a reduction in the immunoreactivity of GFAP, an astrocytic cytoskeletal protein (Bushong et al., 2002), 5 days after surgery. L-AAA-induced reductions in GFAP immunoreactivity was associated with a range of depressive-like behaviours including increased immobility in the forced swim test (FST) 72 h after administration, a decrease in sucrose preference (SPT) at 24 h and 72 h post-surgery, an increase in the latency to feed in the novelty suppressed feeding test (NSFT) at 48 h, and increased escape failures in the active avoidance test (AAT) 4 days following toxin delivery. These depressive-like behaviours and reductions in GFAP-positive cell numbers after L-AAA administration were similar to those induced by CUS (Banasr and Duman, 2008).

In mice, two consecutive doses of L-AAA into the PLC (100 μ g/ μ l, 0.6 μ l, 2 injections) decreased GFAP immunoreactivity 5 days after the second injection. 4 days after L-AAA delivery, a decrease in glutamate and glutamine concentrations was observed in the PFC (including the PLC). However no changes were observed at 2 days post L-AAA administration. Mice with reduced levels of glutamate/glutamine in the PFC also exhibited increased immobility in the FST and a decrease in sucrose preference 4 days after the second L-AAA administration (Lee et al., 2013).

Stereotactically delivered L-AAA is administered through the placement of a guide cannula and subsequent delivery of the toxin locally by microinjection. The procedure therefore requires extensive preparation and the administration of anaesthetic on a number of occasions prior to assessment of the toxin's effect. Moreover 2 consecutive days of administration has been specified in previous reports of L-AAA-induced behaviours changes to glutamate cycling and metabolism in the brain. There are further limitations associated with the model on account of the short window to assess depressive-like behaviours as the effects of the toxin are transient, lasting for a number of days post administration although the temporal profile has not been fully reported to date. In rats, although the authors used a guide cannula, a refinement with a single high dose of L-AAA into the mPFC (20 μ g/ μ l, 5 μ l, 1 injection) resulted in a reduction in the expression of astrocytic cell markers GFAP and S100b, when assessed at 2 and 6 days following L-AAA administration. S100b, a calcium-binding protein,

exerts neurotrophic effect at nanomolar concentration and stimulates neuronal outgrowth whereas higher concentrations may be neurotoxic leading to cell death (Hajduková et al., 2015). L-AAA administration to the mPFC affected performance in the attentional set-shifting test (AASST) 1 day after toxin delivery and in a reversal memory task 2 days post-administration. Impaired working memory in the water-maze was also apparent following L-AAA administration. Performance in these tests are dependent on the mPFC (Lima et al., 2014). Refinement in the L-AAA delivery model, particularly for investigating the effect of the toxin in mice, where a single microinjection without the prior need for placement of a guide cannula in a one-off surgical procedure would confer significant advantages over the model as described in the literature thus far.

GFAP is a cytoskeletal protein principally expressed in the white matter of the brain (Bushong et al., 2002) and is the main intermediate filament in mature astrocytes of the CNS. It is believed to only stain 20-30% of total astrocyte population within a region. GFAP helps maintain astrocyte strength and shape, ultimately playing a key role in astrocyte-neuron communication. However, stress causes a reduction of astrocytic markers such as GFAP and changes astrocytic morphology in the regions of the brain that are predominantly affected in depression (Nestler et al., 2002a). Dysfunctional astrocytes lead to inflammatory responses, BBB impairment, increased neuronal loss, demyelination, and impaired functional recovery following injury (Hu et al., 2016). They release intercellular effector molecules and/or alter gene expression profile, thus influencing surrounding neural cells and influencing axonal and dendritic development, complexity and integrity (Toy and Namgung, 2013). In the L-AAA astroglial toxin model in rats, a high dose of L-AAA (20 μ g/ μ l, 5 μ l, 1 injection) into the PLC decreased NeuN, a marker of mature neurons and increased microglia activation at the injection site 2 and 6 days following L-AAA administration. Iba1 positive cells (microglia), exhibited a typical activated morphology, characterised by a hypertrophic structure (soma) and retracted processes (Lima et al., 2014). In addition, reduced astrocytic function following chronic continuous infusion of L-AAA over 10 days into the left lateral brain ventricle in mice (1 μ g/ μ l L-AAA per hour) counteracts the increase of DCX in the PFC, an immature neuronal marker, following treatment with the hallucinogenic alkaloid harmine (Liu, F., et al., 2017). L-AAA blocked the harmine-induced increase hippocampal neurogenesis.

2. Aims

L-AAA administration seems to act not only by influencing the activity of astroglial cells but also by altering either directly or indirectly microglia and neuronal cells in the CNS.

This study aims to administer the toxin in a once off procedure, therefore reducing the requirement for additional anaesthesia and surgery. Placement of guide cannula leads to prolonged micro and astrogliosis, hence, the proposed refinements may serve to minimise this. Bi-lateral administration of L-AAA (50µg/µl; 1µl) will be administered into the PLC of mice following single or twice daily consecutive doses. Time-course effects of L-AAA will be assessed on both depressive-like behaviour and post-mortem immunohistochemical assessment of astroglia, microglia and neurons in the PLC.

The effect of the impact of sham surgery and anaesthesia on behaviour and astro- and microglial reactivity will also be investigated.

3. Study design

3.1. Surgery

Male C57Bl6/J mice aged around 12-14 weeks old (BioResources, Trinity College Dublin) were anesthetized by IP injection using avertin (1.25g 2,2,2, tribromoethanol; 2.5ml tertiary amyl alcohol; 100ml distilled water) and secured in a stereotactic frame. L-AAA (Sigma Aldrich) (50µg/µl; 1µl; 0.2µl/min) or PBS was administered bilaterally into the PLC (1.7mm anteroposterior, -0.4mm dorsolateral and depth -2.5mm from Bregma) for one or two consecutive days (single versus two consecutive daily doses). Controls were employed to investigate the impact of anaesthesia and surgery. Home cage animals were freely behaving and were not anaesthetised or subjected to surgery. The anaesthesia group was injected with avertin and EMLA cream was applied on the head. Sham surgery (partial surgery) involved administration of the anaesthetic (as described) and the full surgical procedure up to the point of dura puncture.

In a separate experiment, L-AAA (50µg/µl; 1µl; 0.2µl/min) or PBS was administered into the dentate gyrus (DG) (2.2mm anteroposterior, -1.4mm dorsolateral and depth -2.1mm from Bregma). In addition, a qualitative analysis of the distribution of the bolus injection was performed with Evan's blue dye (2% in PBS) following intra-cortical administration [as described for L-AAA (see appendix for further details)].

3.2. Behaviour

All animals were handled daily for five days prior to surgery and behavioural testing (**Fig.1**). 48 h following two consecutive doses of L-AAA, the tail suspension test (TST) was performed. In a separate cohort the splash test (morning) and open-field (OF) (afternoon) were performed.

Following a single dose of L-AAA, four different cohorts were used to assess behaviour. Saccharin preference was recorded for 72 h (0-24 h, 24 h-48 h, 48 h-72 h) following L-AAA administration. Saccharin preference was established during the dark and light phases of the cycle to take advantage of the higher levels of consumption during the dark phase (Harkin et al., 2002). In different cohorts of mice the OF test was performed 24, 48 h and 6 days and TST performed at 48, 72 h or 7 days following L-AAA administration. The FST was performed at 48 and 72 h post-L-AAA. Controls were employed to assess the impact of anaesthesia and surgery on behaviour (FST, TST, saccharin preference, OF and splash test)

48-72 h following administration of anaesthetic and/or exposure to the sham surgery procedures.

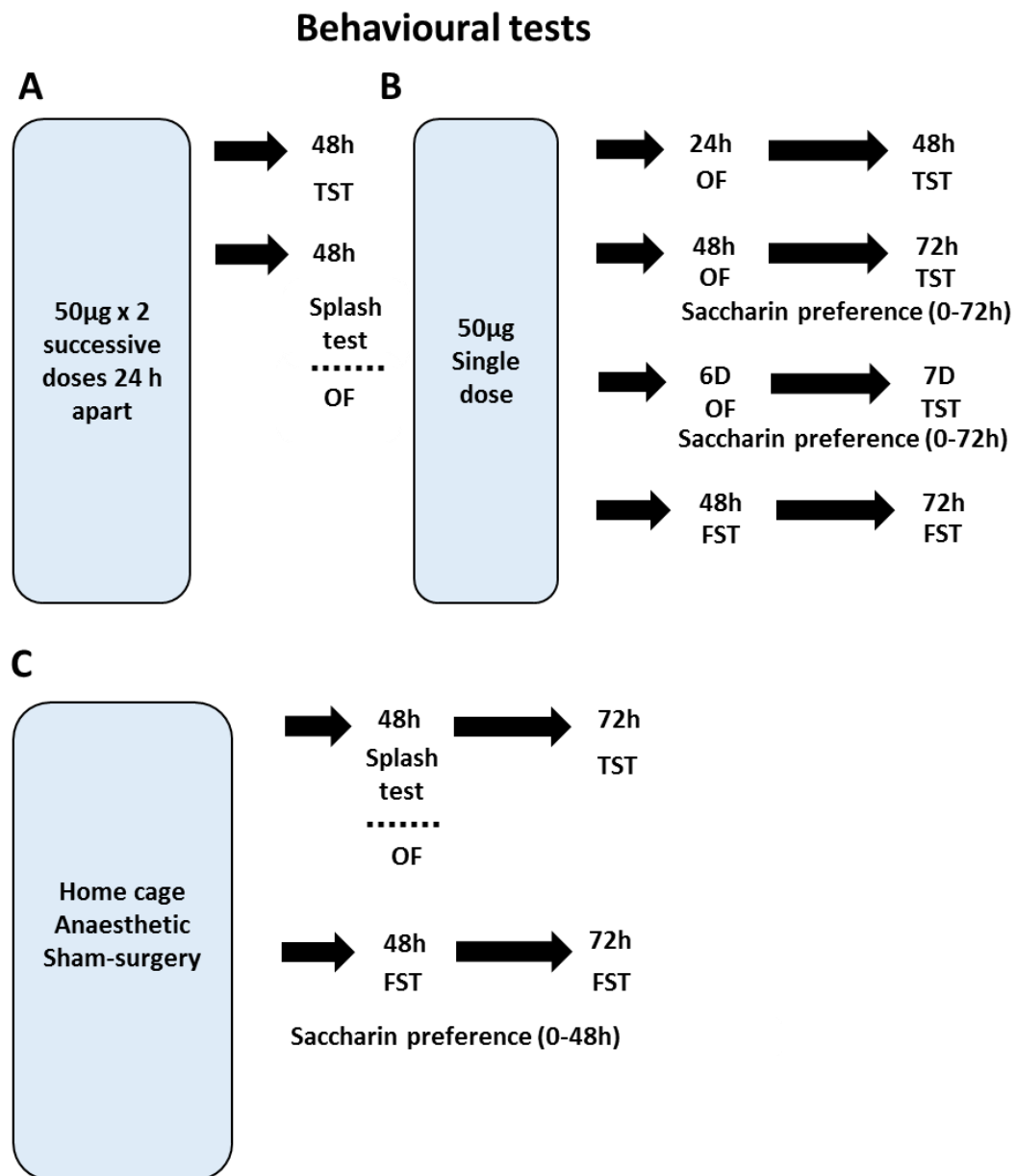


Figure 4.1: Behavioural testing

A) Mice received two successive injections of L-AAA (50µg/µl; 1µl, 24 h apart) or PBS followed by the TST 48 h after L-AAA administration. In a separate cohort the splash test (morning) and OF (afternoon) were performed 48 h after L-AAA administration. **B)** After a single L-AAA administration (50µg/µl; 1µl), four different cohorts were used to assess the behaviour. After surgery, saccharin preference was measured over 72 h (0-24 h, 24 h-48 h, 48 h-72 h). OF was performed at 24, 48 h and 6 days and TST was performed at 48, 72 h or 7 days in different cohorts following L-AAA administration. The FST was performed at 48 and 72 h post-L-AAA. **C)** Controls were employed to investigate the effect of anaesthesia and surgery on behaviour. Two cohorts were used to assess the behaviour. Splash test (morning) and OF (afternoon) were performed at 48 h and TST was performed at 72 h following surgery/control surgery. Another cohort was used to investigate the effect of the surgery on saccharin preference and FST at 48 and 72 h post-surgery.

3.3. Post-mortem analysis

Post mortem studies were performed at different time points, to link to behavioural outcomes and assess the effect L-AAA on astrocytes (GFAP, S100b), microglia (Iba1) and neurons (NeuN and DCX). Mice were first transcardially perfused with heparinised PBS (0.1 M, pH 7.4; heparin 1ml at 5000UI/ml) for 3 min, and then with PFA (4% PFA in PBS) for 3 min 30 seconds with the abdomen clamped. Brains were then removed from the skull and placed in 4% PFA overnight, followed subsequently by placement in 30% sucrose solution (w/v) for 3 days. After these steps, brains were stored in a freezing solution at -80 degrees until sectioning.

Sections from the PLC were incubated with rabbit polyclonal anti glial fibrillary acid protein antibody (GFAP 1:1000, DAKO) or rabbit S100 calcium-binding protein B (S100b 1: 1000, Abcam) with anti-rabbit secondary antibody (1:1000 Alexa 488nm, Invitrogen) to visualise astrocytes. To visualise microglia, sections were incubated with rabbit polyclonal anti-ionized calcium-binding adapter molecule 1 (Iba1 1:3000, Vectastain) and positive signals were developed using Elite vector ABC kit (Vector laboratories) and 3,3'-Diaminobenzidine (DAB) as a substrate for peroxidase. To visualise mature neurons, sections from the PLC were incubated with mice monoclonal anti-NeuN (1:300, Millipore) and anti-mouse (1:500 Alexa 488nm, Invitrogen). Sections from the hippocampal DG were incubated with rabbit polyclonal anti GFAP (1:1000, DAKO) with anti-rabbit secondary antibody (1:1000 Alexa 546nm, Invitrogen) to visualise astrocytes and mouse anti DCX (1:250, Santa Cruz) with an anti-mouse secondary antibody (1:1000 Alexa 488nm Invitrogen) to visualise immature neurons. (See chapter 2 material and methods for further details)

4. Statistical analysis

Data are expressed as group mean with SEM and were analysed using unpaired Student's *t*-tests or analysis of variance (ANOVA). If any statistically significant change was found following one or two factor ANOVA, *post-hoc* comparisons were performed using a Fisher's LSD test. Data were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

5. Results

5.1. Behaviour

5.1.1. Effect of intra-cortical L-AAA administration on bodyweight

Two-way repeated measure ANOVA of bodyweight showed an effect of time following two consecutive L-AAA doses [$F_{(3,45)} = 68.08$, $P < 0.0001$], no effect of L-AAA [$F_{(1,15)} = 0.3138$, $P = 0.5836$], and no L-AAA x time interaction [$F_{(3,45)} = 0.3557$, $P = 0.7852$] (data not shown). Two-way repeated measure ANOVA of bodyweight showed an effect of time after a single L-AAA dose [$F_{(3,48)} = 48$, $P < 0.0001$], no effect of L-AAA [$F_{(1,16)} = 0.2754$, $P = 0.6069$], and no L-AAA x time interaction [$F_{(3,48)} = 2.316$, $P = 0.0876$] (data not shown).

5.1.2. Effect of two consecutive daily doses of L-AAA in behavioural tasks

L-AAA administration decreased grooming behaviour (back licking) in the splash test. Two-way repeated measure ANOVA showed an effect of time on grooming behaviour in the splash test [$F_{(1,10)} = 21.64$, $P < 0.001$], an effect of L-AAA [$F_{(1,10)} = 6.922$, $P = 0.0251$] but no L-AAA x time interaction [$F_{(1,10)} = 2.318$, $P = 0.1588$]. Post-hoc comparisons revealed that L-AAA administration decreased grooming behaviour 5 to 10 min following application of sucrose when compared to vehicle-treated controls [$P < 0.05$] (**Fig 4.2.A**).

The effect of L-AAA on immobility in the TST is shown in **Fig 4.2.B**. Student's T-test showed that L-AAA increases immobility in the TST 48 h after administration [$T_{(17)} = 2.274$, $P = 0.0362$] when compared to vehicle-treated controls. Student's T-test showed no effect of L-AAA administration on exploration (percentage of time spent in the peripheral area) and locomotion in the OF 48 h post-surgery when compared to vehicle-treated controls. [$T_{(18)} = 0.2175$, $P = 0.8303$] and [$T_{(18)} = 0.7130$; $P = 0.4850$] (data not shown).

5.1.3. Effect of a single dose of L-AAA in behavioural tasks

The effect of L-AAA administration on saccharin preference is shown in **Fig 4.2.C**. Two-way repeated measure ANOVA of saccharin preference showed an effect of time [$F_{(3,72)} = 16.43$, $P < 0.001$], an effect of L-AAA [$F_{(1,24)} = 4.409$, $P = 0.0464$], but no L-AAA x time interaction [$F_{(3,72)} = 1.640$, $P = 0.1877$]. Post hoc comparisons revealed that L-AAA administration decreased saccharin preference over the initial 24 h period following L-AAA administration when compared to vehicle-treated controls [$P < 0.01$]. Two-way repeated measure ANOVA on total fluid consumption showed an effect of time [$F_{(3,72)} = 5.368$, $P = 0.0022$], no effect of L-

AAA, [$F_{(1,24)} = 0.1953$, $P=0.6625$] and no L-AAA x time interaction [$F_{(3,72)} = 0.6543$, $P=0.5829$] (data not shown).

L-AAA didn't affect exploration (percentage of time spent in the peripheral area) and locomotor activity in the OF at 24, 48 h and 6 days after L-AAA administration. Two-way ANOVA of time spent in the peripheral area in the open field showed no effect of time [$F_{(2,51)} = 1.339$; $P=0.2710$], no effect of L-AAA treatment [$F_{(1,51)} = 0.3272$, $P=0.5698$] or a time x L-AAA interaction [$F_{(2,51)} = 1.566$, $P=0.2187$]. Two-way ANOVA of total distance travelled showed an effect of time on total locomotion in the OF [$F_{(2,49)} = 3.335$; $P=0.0439$], no effect of L-AAA treatment [$F_{(1,49)} = 0.05134$, $P=0.8217$] but a L-AAA x time interaction [$F_{(2,49)} = 3.437$, $P=0.0401$] (data not shown).

L-AAA increased immobility in the TST 48 h after L-AAA administration with a trend towards increased immobility also evident at 72 h but no effect observed 7 days following L-AAA administration compared to vehicle-treated controls (**Fig 4.2.C**). Two-way ANOVA showed no effect of time on the immobility in the TST [$F_{(2,50)} = 1.146$; $P=0.32$], an effect of L-AAA administration [$F_{(1,50)} = 8.26$, $P=0.0059$], but no interaction between L-AAA x time [$F_{(2,50)} = 0.19$, $P=0.826$]. Post hoc comparisons revealed that L-AAA increased the immobility in the TST when compared to vehicle-treated controls 48 h post L-AAA administration [$P<0.05$] (**Fig 4.2.D**).

Two-way repeated measure ANOVA of immobility in the FST showed no effect of time [$F_{(1,16)} = 0.9097$; $P=0.3544$], an effect of L-AAA administration [$F_{(1,16)} = 6.494$, $P=0.0215$], but no interaction between L-AAA x time [$F_{(2,16)} = 0.06116$, $P=0.8078$]. Post hoc comparisons revealed that L-AAA increased the immobility in the FST when compared to vehicle-treated controls 48 h post L-AAA administration [$P<0.05$] (**Fig 4.2.E**).

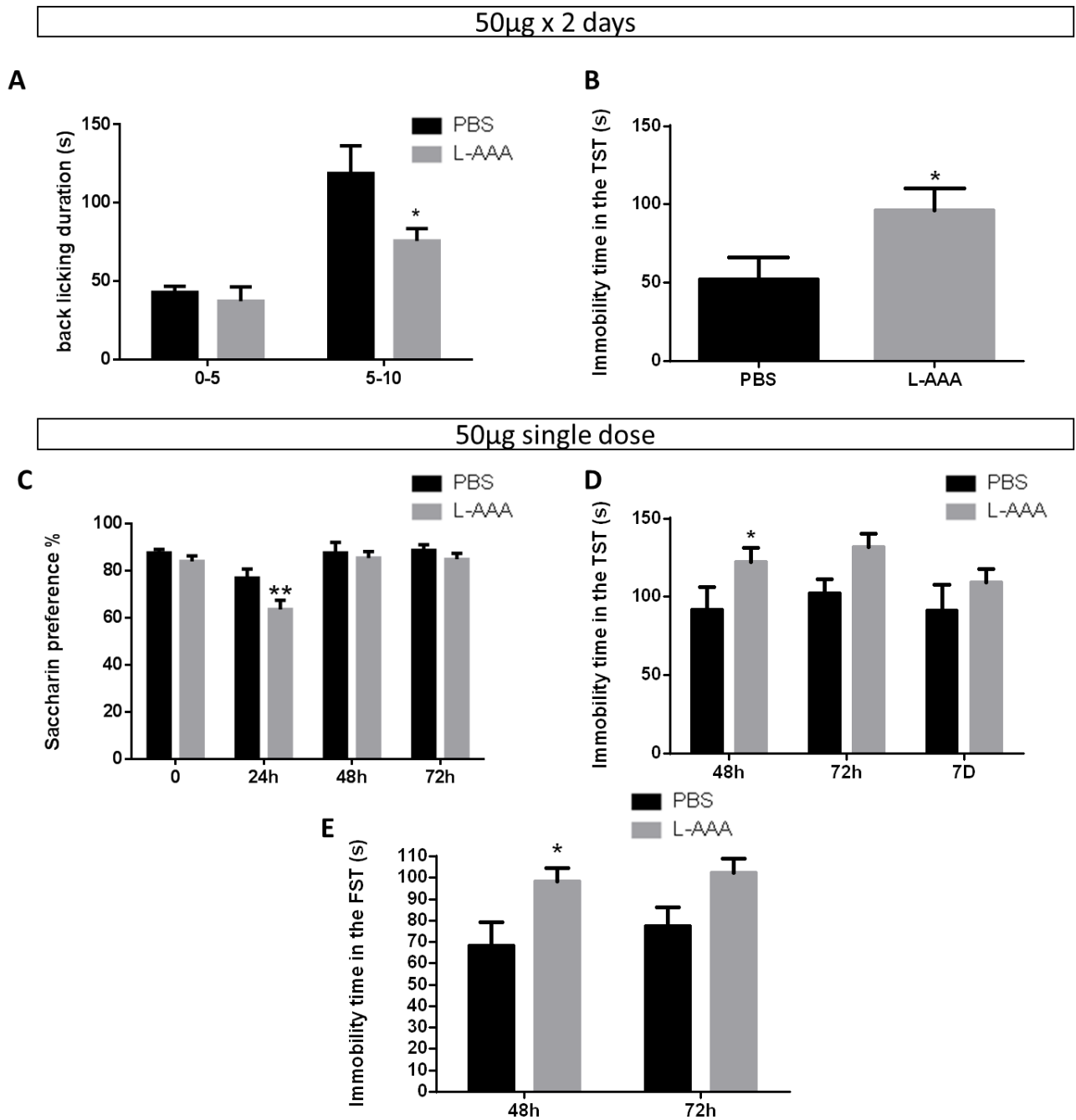


Figure 4.2: Effect of a single or two successive L-AAA doses on behaviour

Mice received two bilateral injections into the PLC (50µg/µl; 1µl, 24 h apart). L-AAA administration decreased grooming behaviour at 48 h (PBS; N=6; L-AAA; N=6) **A**) and increased immobility in the TST at 48 h (PBS; N=10; L-AAA; N=9) **B**). After a single bilateral injection into the PLC (50µg/µl; 1µl), behaviour was assessed at different time points over a week following administration of L-AAA in different groups. L-AAA administration decreased saccharin preference (PBS; N=12; L-AAA; N=14) **C**), increased immobility in the TST at 48 h (PBS; N=10; L-AAA; N=10) **D**) with a trend towards increase at 72 h (PBS; N=10; L-AAA; N=9) but no effect at 7 days (PBS; N=7; L-AAA; N=10) **D**). L-AAA increased immobility in the FST over the last 4 min (2 to 6 min) at 48 h with a trend towards increase at 72 h post administration (PBS; N=10; L-AAA; N=8) **E**). Data are expressed as mean $\pm$ SEM, *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ for L-AAA versus vehicle-treated controls.

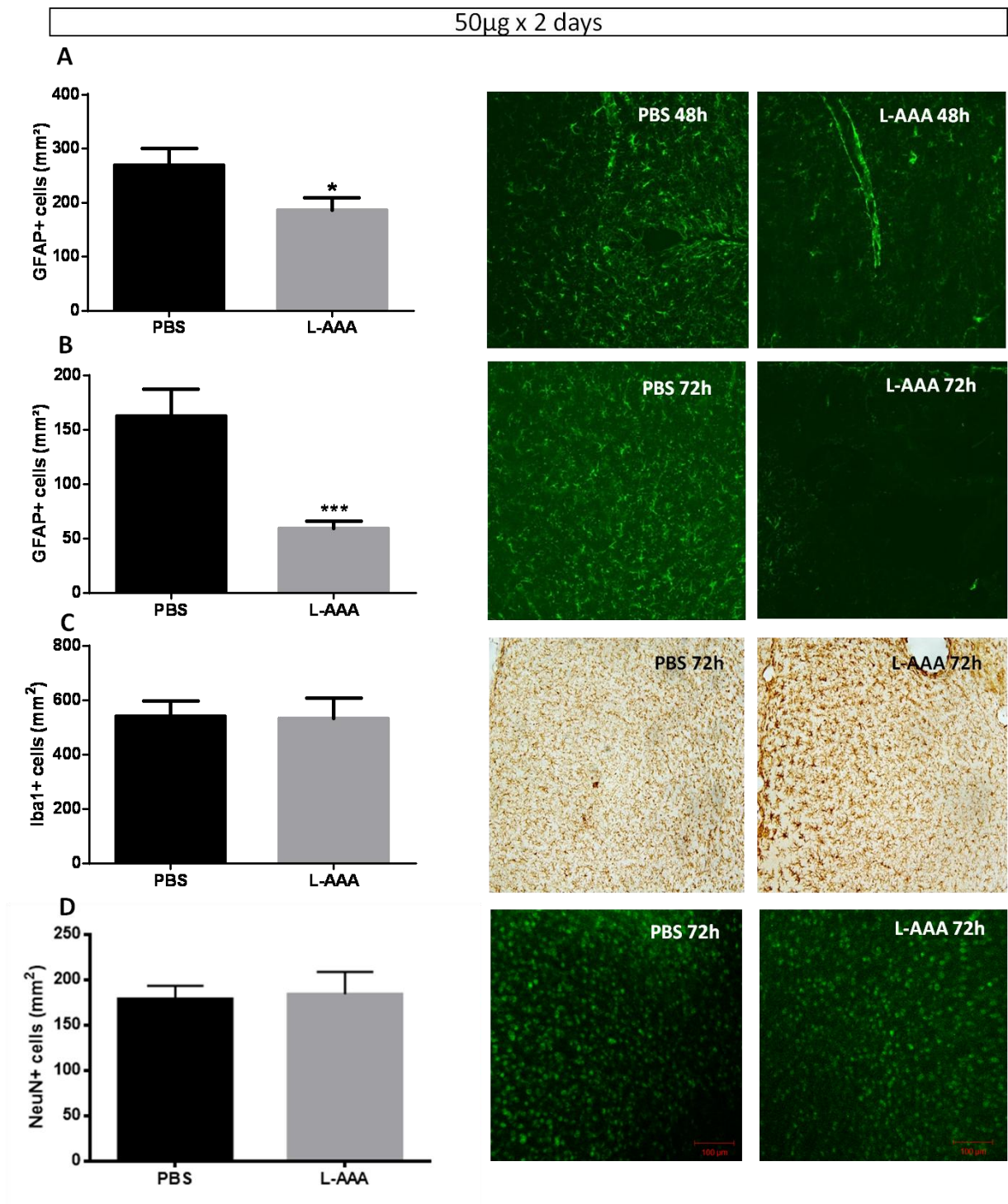
5.2. Post-mortem analysis

5.2.1. Effect of two consecutive daily doses of L-AAA on GFAP, IBA1 and NeuN immunoreactivity in the PLC

Student's T-test showed that L-AAA administration decreases GFAP immunoreactivity in the PLC 48 h after L-AAA administration, [$T_{(16)}=2.197$, $P=0.0431$] when compared to vehicle-treated controls (**Fig 4.3.A**). L-AAA didn't affect GFAP immunoreactivity in the areas peripheral to the injection site (data not shown). Quantitative analysis of GFAP immunoreactivity 72 h following intra-cortical L-AAA administration is shown in the **Fig 4.3.B**. Student's T-test showed that L-AAA administration decreases GFAP immunoreactivity 72 h post administration of L-AAA [$T_{(17)}=4.195$, $P=0.0006$]. Student's T-test showed no effect of L-AAA administration on Iba1 immunoreactivity 72 h after L-AAA administration [$T_{(4)}=0.09022$, $P=0.9324$] when compared to vehicle-treated controls (**Fig 4.3.C**). Similarly there was no effect on NeuN immunoreactivity 72 h post L-AAA administration in the mouse PLC [$T_{(14)}=0.1674$; $P=0.8694$] (**Fig 4.3.D**)

5.2.2. Effect of a single dose of L-AAA on GFAP, S100b and NeuN immunoreactivity in the PLC

Analysis of GFAP immunoreactivity following intra-cortical L-AAA administration is shown in **Fig 4.3.E**. Two-way ANOVA of GFAP immunoreactivity showed an effect of time [$F_{(2,49)}=5.742$; $P=0.0058$], an effect of L-AAA [$F_{(1,49)}=9.522$, $P=0.0033$], but no interaction between L-AAA x time [$F_{(2,49)}=0.06116$, $P=2.977$]. Post hoc comparisons revealed that L-AAA decreased GFAP positive cells when compared to vehicle-treated controls at 48 h [$P<0.01$] and 72 h [$P<0.05$] post L-AAA administration. L-AAA also decreased S100b 72 h following administration when compared to vehicle treated controls [$T_{(15)}=2.544$; $P=0.02$] (**Fig 4.3.F**). There was no effect on NeuN immunoreactivity 72 h post L-AAA administration in the mouse PLC [$T_{(15)}=0.1297$; $P=0.8985$] (**Fig 4.3.G**) or after a single bilateral infusion of 100 μ g/ μ l L-AAA administration [$T_{(17)}=1.457$; $P=0.1633$] (data not shown).



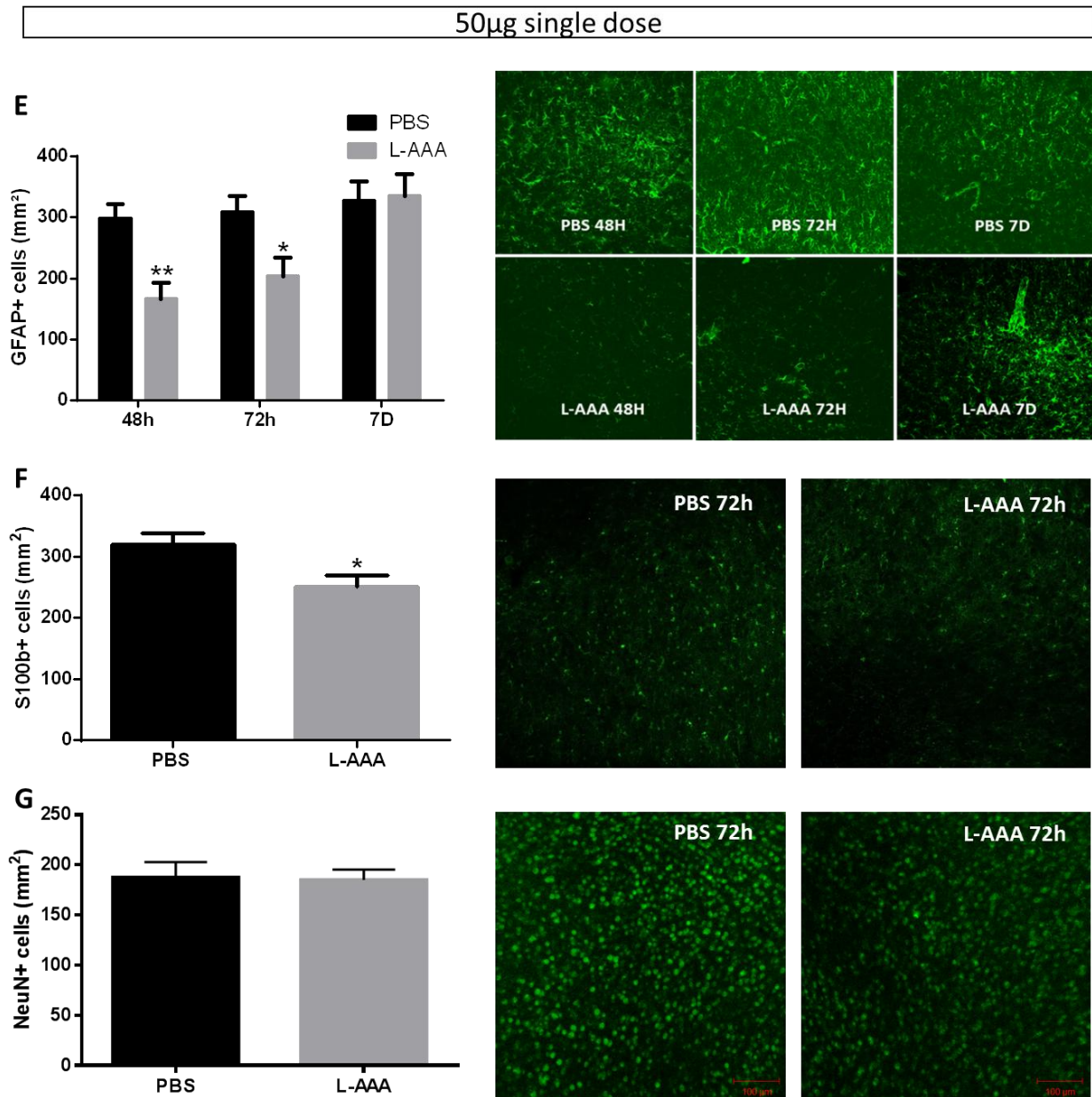


Figure 4.3: Investigation of the effect a single or two successive L-AAA doses on immunoreactivity of GFAP, IBA1 and NeuN in the PLC

Mice received two bilateral injections into the PLC (50µg/µl; 1µl, 24 h apart). L-AAA decreased GFAP at 48 h (PBS; N=9; L-AAA; N=9) **A**) and 72 h (PBS; N=9; L-AAA; N=10) **B**) but did not affect Iba1 immunoreactivity in the PLC at 72 h (PBS; N=3; L-AAA; N=3) **C**). L-AAA did not affect NeuN (PBS; N=7, L-AAA; N=9) **D**).

After a single bilateral injection into the PLC (50µg/µl; 1µl), there were a decreased GFAP immunoreactivity in the PLC at 48 (PBS; N=8; L-AAA; N=10) and 72 h (PBS; N=10; L-AAA; N=9) with no effect after 7 days (PBS; N=8; L-AAA; N=10) **E**). L-AAA decreased S100b immunoreactivity 72 h after injection (PBS; N=9; L-AAA; N=7) **F**) but not NeuN immunoreactivity (PBS; N=10; L-AAA; N=7) **G**) Data are expressed as mean ± SEM, *, P<0.05; **, P<0.01, ***, P<0.001 for L-AAA versus vehicle-treated controls.

5.3. Effect of surgery and anaesthesia

5.3.1. Effect of surgery and anaesthesia on bodyweight

Surgery, sham surgery and anaesthesia produced a decrease in body weight. Two-way repeated measure ANOVA of bodyweight showed an effect of time [$F_{(3,99)} = 101$, $P < 0.001$], an effect of procedure [$F_{(3,33)} = 4.432$; $P = 0.01$] and a procedure x time interaction [$F_{(9,99)} = 11.34$, $P < 0.001$]. Post hoc comparisons revealed that anaesthesia decreased bodyweight at 24 h [$P < 0.05$], 48 h [$P < 0.001$] and 72 h [$P < 0.001$] when compared to home cage controls. Sham surgery decreased bodyweight when compared to home cage controls at 24 h [$P < 0.05$], 48 h [$P < 0.001$] and 72 h [$P < 0.01$]. PBS administration decreased bodyweight when compared to home cage controls at 24 h [$P < 0.01$], 48 h [$P < 0.001$], 72 h post-surgery [$P < 0.001$]. All groups lost weight but there were no weight differences between the sham/complete surgery and the anaesthesia treated groups (**Fig 4.4.A**).

5.3.2. Effect of surgery and anaesthesia on behavioural tasks

Two-way repeated measure ANOVA of saccharin preference showed an effect of time [$F_{(2,62)} = 6.861$, $P = 0.0020$], no effect of procedure [$F_{(3,31)} = 2.849$, $P = 0.0535$], but a procedure x time interaction [$F_{(6,62)} = 3.924$, $P = 0.0022$]. Post hoc comparisons revealed that anaesthesia, sham surgery and PBS administration decreased the saccharin preference over the 24 h period [$P < 0.05$], and from 24 h-48 h after surgery [$P < 0.01$] compared to home cage controls (**Fig 4.4.B**). Two-way repeated measure ANOVA of total fluid consumption showed an effect of time [$F_{(2,62)} = 11.87$, $P < 0.001$], no effect of procedure [$F_{(3,31)} = 0.3587$, $P = 0.7832$], no procedure x time interaction [$F_{(6,62)} = 0.916$, $P = 0.4857$] (data not shown).

Two-way repeated measure ANOVA of saccharin consumption showed an effect of time [$F_{(2,62)} = 3.219$, $P = 0.0468$], no effect of procedure [$F_{(3,31)} = 1.380$, $P = 0.2672$], no procedure x time interaction [$F_{(6,62)} = 1.078$, $P = 0.3852$] (data not shown). Two-way repeated measure ANOVA of water consumption showed an effect of time [$F_{(2,62)} = 12.98$, $P < 0.001$], an effect of procedure [$F_{(3,31)} = 3.303$, $P = 0.0331$], a procedure x time interaction [$F_{(6,62)} = 2.284$, $P = 0.0468$]. Post hoc comparisons revealed that sham surgery increased water consumption over the 24 h period [$P < 0.05$] and that sham surgery [$P < 0.001$], complete surgery and anaesthesia [$P < 0.05$] increased water consumption from 24 to 48 h after injection compared to home cage controls (**Fig 4.4.C**).

Two-way repeated measure ANOVA of time spent grooming (back licking) showed an effect of time [$F_{(1,28)} = 51.05$, $P < 0.001$], an effect of procedure [$F_{(3,28)} = 5.496$, $P < 0.001$], and a

procedure x time interaction [$F_{(3,28)} = 3.762$, $P = 0.0218$]. Post hoc comparisons revealed that anaesthesia [$P < 0.01$], sham surgery and PBS administration [$P < 0.001$] increased grooming duration 5-10min following sucrose application 48 h after surgery compared to the home cage controls (**Fig 4.4.D**).

One way ANOVA of percentage of time spent in the peripheral area in the open field showed no effect of procedure [$F_{(3,32)} = 2.599$, $P = 0.0693$] or total distance travelled in the OF [$F_{(3,34)} = 1.142$, $P = 0.3461$] compared to home cage controls (data not shown).

One-way ANOVA of immobility in the TST showed no effect of procedure [$F_{(3,35)} = 2.374$, $P = 0.0868$] (**Fig 4.4.E**). Two-way repeated measure ANOVA of immobility in the FST showed no effect of time in the FST [$F_{(1,32)} = 1.337$, $P = 0.2561$], no effect of procedure [$F_{(3,32)} = 1.895$, $P = 0.1504$], and no procedure x time interaction [$F_{(3,32)} = 0.1446$; $P = 0.9324$] compared to home cage controls (**Fig 4.4.F**), indicating that neither surgery nor anaesthesia affected immobility in the TST or FST.

5.3.3. Effect of surgery and anaesthesia on GFAP and Iba1 immunoreactivity in the PLC

Post mortem analysis of GFAP immunoreactivity in the PLC is shown in **Fig 4.5.A**. One way ANOVA of GFAP immunoreactivity showed an effect of procedure [$F_{(3,35)} = 30.74$, $P < 0.001$]. Post hoc comparisons revealed that PBS injection increased GFAP immunoreactivity in the PLC when compared to home cage control [$P < 0.001$] 72 h post-surgery. These results suggest that surgery (with micro capillary injection of PBS) but not anaesthesia increased GFAP immunoreactivity in the PLC.

Analysis of Iba1 immunoreactivity in the PLC is shown in **Fig 4.5.B**. One-way ANOVA of Iba1 immunoreactivity showed an effect of procedure [$F_{(3,8)} = 6.254$, $P = 0.0171$]. Post hoc comparisons revealed that PBS administration increased Iba1 immunoreactivity when compared to home cage controls [$P < 0.01$] 72 h post-surgery. These results suggest that surgical placement of the microcapillary and/or delivery of PBS, but not anaesthesia or surgical manipulation apart from the injection procedure, increased Iba1 immunoreactivity.

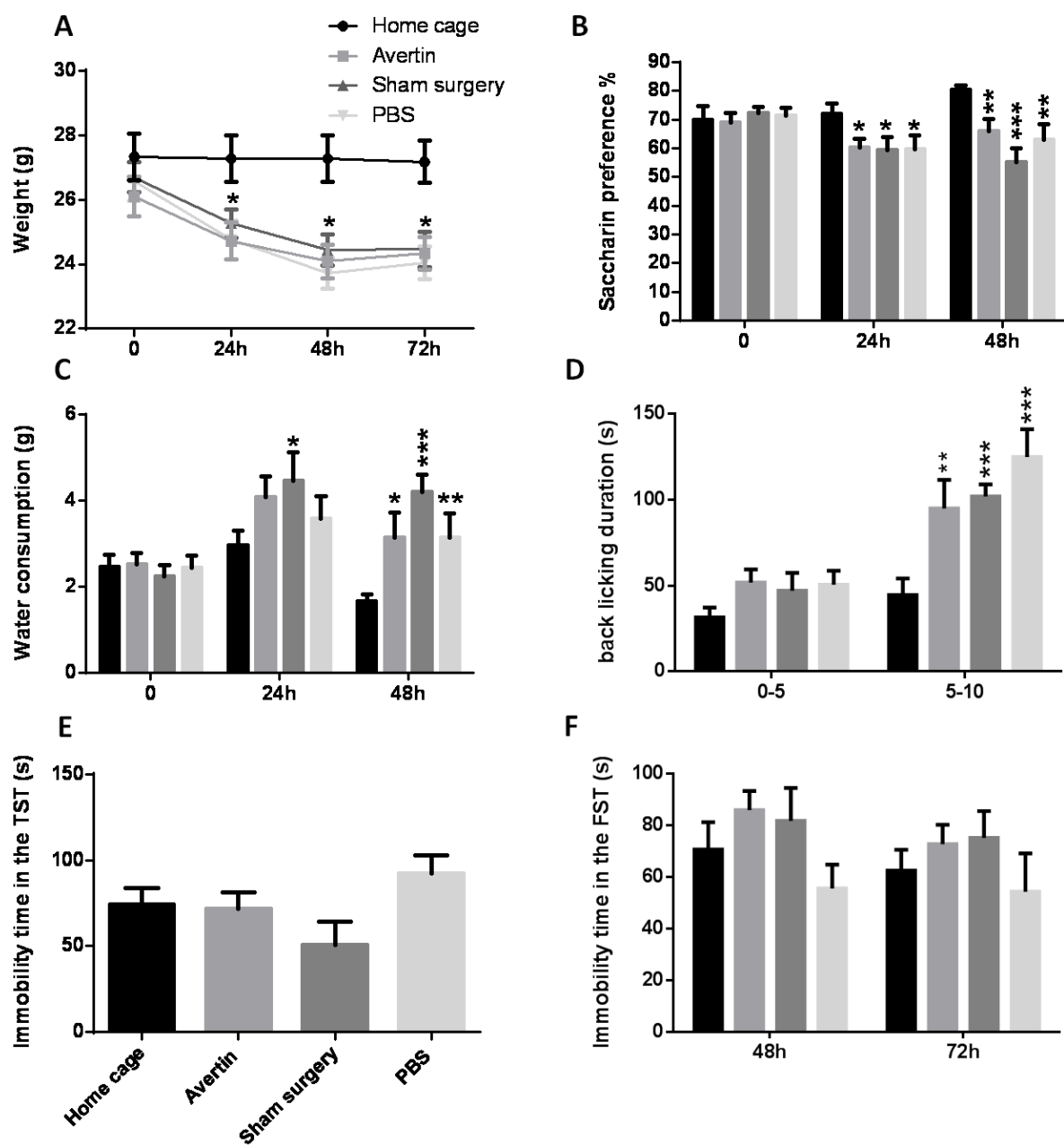


Figure 4.4: Investigation of the impact of surgery and anaesthesia on behaviour

Controls were used to assess the effect of the anaesthesia and surgery on bodyweight compared to PBS and L-AAA-treated groups. Surgery (PBS), sham surgery or anaesthesia decreased the bodyweight. (Home cage; N=10, Avertin; N=10, Sham surgery; N=9; PBS; N=8), **A**), reduced saccharin preference **B**) and increased water consumption **C**) (Home cage; N=9, Avertin; N=10, Sham surgery; N=8; PBS; N=8). Surgery and anaesthesia increased grooming behaviour indicating by back licking duration (Home cage; N=8, Avertin; N=9, Sham surgery; N=8; PBS; N=7) **D**). There was no differences between the groups on immobility in the TST (Home cage; N=10, Avertin; N=10, Sham surgery; N=10; PBS; N=9) **E**) or the FST (Home cage; N=10, Avertin; N=10, Sham surgery; N=9; PBS; N=7) **F**). Data are expressed as mean $\pm$ SEM, *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ for all groups versus home cage controls.

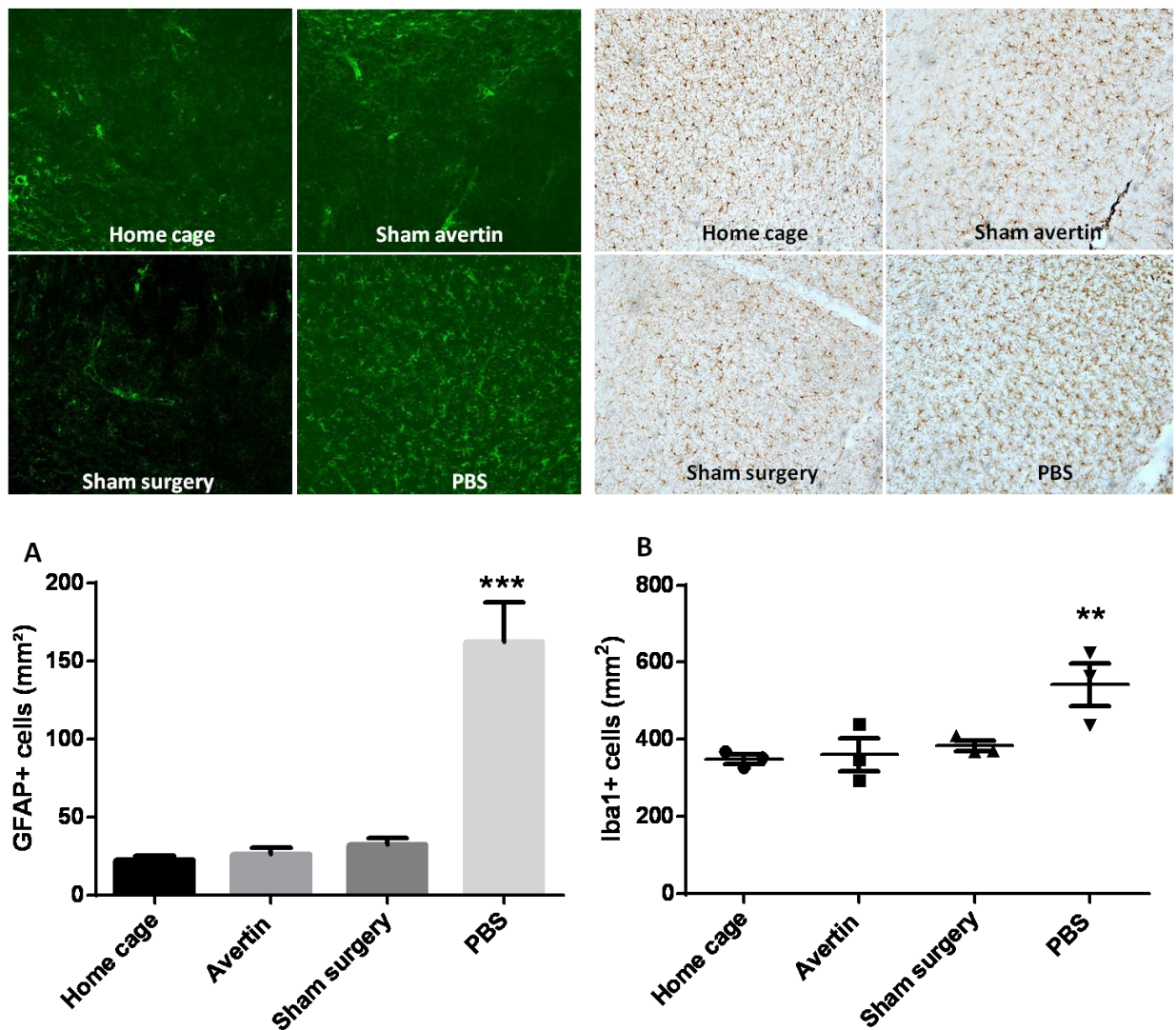


Figure 4.5: Investigation of the impact of surgery and anaesthesia on GFAP and Iba1 immunoreactivity in the PLC

Controls were used to assess the impact of the anaesthesia and surgery on both behaviour and GFAP and Iba1 immunoreactivity in the PLC. **A)** Surgery increased GFAP immunoreactivity in the PLC 72 h post L-AAA administration (Home cage; N=10, Avertin; N=10, Sham surgery; N=10; PBS; N=9). **B)** Surgery increased Iba1 immunoreactivity 72 h post L-AAA administration (Home cage; N=3, Avertin; N=3, Sham surgery; N=3; PBS; N=3). Data are expressed as mean $\pm$ SEM, *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ versus home cage controls.

6. Discussion

The overarching aim of this study was to refine a mouse model of cortical astrocytic impairment leading to depressive-related behaviours. Results indicate that while a depressive behavioural phenotype and reduced cortical GFAP immunoreactivity are observed following two consecutive daily doses of L-AAA (50µg/ul; 1µl; 2 days), a single dose (50µg/ul; 1µl) was also sufficient to induce both depressive-like behaviours and reduced GFAP immunoreactivity. Following L-AAA administration, a transient depressive-like behaviour and reduced cortical GFAP immunoreactivity was evident over a 72 h post administrative period leading to a recovery from behavioural deficit and reduced GFAP immunoreactivity over 7 days. In addition to reduced GFAP, a reduction in the immunoreactivity of the astrocytic protein s100b was observed in the PLC following a single dose of L-AAA to the PLC without affecting NeuN or Iba1 immunoreactivity.

6.1. Effect of L-AAA in behavioural tasks

Both single and double doses (50µg/ul; 1µl) of L-AAA had no effects on bodyweight when compared to vehicle treated controls. Anhedonia and apathy were measured using the saccharin preference and sucrose splash tests. Results showed that a single dose of L-AAA reduced saccharin preference over the 24 h period following L-AAA administration in a similar way to that observed in the sucrose preference test 24 h post L-AAA administration in rats (100µg/ul; 0.5µl; 2 days) (Banasr and Duman, 2008) or 4 days after L-AAA administrations in mice (100µg/ul; 0.6µl; 2 days) (Lee et al., 2013). 2 doses of L-AAA (50µg/ul; 1µl; 2 days) decreased grooming duration (back licking) in the splash test 48 h after L-AAA administration. These findings are in line with those observed with the CUS model, a rodent model of depression (Yalcin et al., 2005) to indicate that L-AAA administration to the PLC in mice can produce anhedonic and apathy-related behaviours for a time depending on the dose regimen.

Observations from the OF test indicate that L-AAA does not provoke anxiety-like behaviours or impairments in exploratory/locomotor activity following either a single or double dose. Similar results were reported in mice 4 days after L-AAA administrations (Lee et al., 2013). However, 1 or 2 L-AAA doses to the PLC increased immobility in the TST 48 h after L-AAA administration as shown previously 24 h after 10 days of L-AAA administration (1 µg/µl L-AAA per hour in the left lateral brain ventricle) (Liu F., et al., 2017). There was a trend

towards increased immobility in the TST 72 h after a single dose of L-AAA with no change in immobility indicative of a recovery achieved one week following administration.

A single dose of L-AAA to the PLC increased immobility 48 h post-surgery in the FST with a trend towards increase after 72 h. These findings are similar to the effects of L-AAA reported in the rat model 72 h after a second L-AAA dose and to those effects observed with the CUS depression model (Banasr and Duman, 2008). Increased immobility in the FST was also observed in mice 48 h after 10 days of L-AAA continuous infusion in the left lateral brain ventricle (Liu F., et al., 2017). Another study demonstrated an increase in immobility 4 days after two daily doses of L-AAA in the PLC with a recovery achieved 9 days post administration (Lee et al., 2013).

6.2. Effect of L-AAA on GFAP, S100b, Iba1 and NeuN immuno-reactivity

Both single and 2 consecutive daily doses of L-AAA produced a reduction in GFAP immunoreactivity 48 h and 72 h post administration when compared to vehicle treated controls. Recovery from reduced GFAP immunoreactivity was evident 7 days following L-AAA administration. Those findings are similar to those previously described in rats 5 days after L-AAA administration (Banasr and Duman, 2008) or 48 h and 6 days after L-AAA administration in rats (Lima et al., 2014) or 5 days after the second administration in mice (Lee et al., 2013).

In addition, a single dose of L-AAA induced a decrease in S100b immunoreactivity when assessed 72 h after administration. Similar results were observed 48 h after a single L-AAA injection in the PLC in rats (Lima et al., 2014). Circulating plasma concentrations of S100b are reported to be increased in mood disorders, particularly in depression (review Schroeter et al., 2013). S100b is altered in both serum and cerebrospinal fluid of patients with mood disorders (Grabe et al., 2001; Schroeter et al., 2008). Severity of depression had a significantly positive association with S100b levels (Tsai and Huang, 2016). The levels of the astrocytic protein S100b have been found to be specifically altered in the lateral PFC and parietal cortices in bipolar disorders (BD), with a decrease in Brodmann's area 9 and increase in Brodmann's area 40 (Dean et al., 2006) and decrease in the CA1 pyramidal layer of MDD and BD patients (Gos et al., 2013). The present results suggest that L-AAA may produce a cytoskeletal impairment (GFAP reduction), contributing to changes in BBB permeability which could help account for alterations in circulating S100b concentrations released by astrocytes with a decrease of S100b in the brain and an increase in S100b levels in the

plasma. Measuring the circulating level of S100b in the L-AAA model would help to understand the circulating changes observed in depressed patients.

Furthermore, the lack of changes observed in Iba1 and NeuN immunoreactivity at 72 h following a single or two consecutive doses of L-AAA may suggest that behavioural changes are a result of astrocytic dysfunction and not due to loss of post-mitotic neurons or inflammatory reactions related to an increase in the number of microglia. However, those cells seem to present a reactive profile (hypertrophy of the soma and retracted processes as previously reported following L-AAA administration (Lima et al., 2014) indicative of an activation of microglia that might affect astrocytes (not quantified). In addition, maximal behavioural effects are observed at 24-48 h timepoints and not at 72 h. This is a limitation of this study as all the animals used for the control experiments were euthanised at 72 h. Indeed on P160 of your discussion, you conclude “lack of changes observed in iba-1 and NeuN immunoreactivity at 72 h following a single or two consecutive doses of L-AAA suggests that behavioural changes are a result of astrocytic dysfunction and not due to loss of post-mitotic neurons or inflammation related to microglia” A more tempered conclusion required here; behavioural changes happen at 24-48 h not at 72 hrs and you did not differentiate between resting state and activated microglia. These results are in line with Banasr and Duman (2008) who found no effect on NeuN immunoreactivity 5 days after 2 L-AAA injections, although there were signs of cell stress, including the presence of vacuoles and increased neuronal cell body size. The lack of effect of L-AAA on NeuN immunoreactivity differs from a previous report (Lima et al., 2014) that showed that a single high dose of L-AAA (20µg/µl, 5µl, 1 injection) decreased NeuN immunoreactivity 2 and 6 days following L-AAA administration. They also showed that microglia exhibited a typically activated morphology, characterized by hypertrophic structure with retracted processes at the injection site but the number of microglia was not quantified.

6.3. Effect of surgery and anaesthesia in behavioural tasks

To clarify that the effects of L-AAA on behaviour were related to changes in astrocytic activation rather than procedures related to the delivery of the toxin, the impact of anaesthesia and surgery was assessed on performance in the behavioural tests. Surgery (PBS injection), sham surgery and anaesthesia, all had a similar effect on bodyweight. All groups exhibited a reduction in saccharin preference from 0-24 h and 24-48 h post-anaesthetic or surgical manipulation probably associated to avertin administration. No difference in total fluid

consumption or saccharin consumption was observed between the groups. However, an increase in water consumption was observed which is indicative of recovery from dehydration related to the impact of the anaesthetic and recovery from the surgical procedure. Although surgery decreased saccharin preference, the decrease of saccharin preference associated with L-AAA was more pronounced suggesting an effect of L-AAA on anhedonia-like behaviour. Results further demonstrated a post-operative stress response associated with increased grooming duration (back licking). Delivery of the anaesthetic or exposure to surgery had no effect on exploratory or locomotor activity in the OF, and no effect on the immobility measured in both TST and FST when compared to home cage controls. These results suggest that after anaesthesia, the animals are drinking more water than saccharin and present an increase heightened self-care response commensurate with recovery from surgical procedure. Furthermore, as no changes in immobility in the TST and FST were observed between groups compared to PBS controls (surgery control group), it is possible to conclude that the increase in immobility following L-AAA administration is linked to administration of the toxin rather than an impact of the surgical procedure.

6.4. Effect of surgery and anaesthesia on GFAP and Iba1 immunoreactivity

To determine the specificity of L-AAA, the impact of anaesthesia and the surgical procedure was investigated. These results suggest that surgical placement of the micro capillary tube and/or PBS administration induced GFAP and Iba1 immunoreactivity in the PLC, while anaesthesia had no effect. These results are not unexpected and in line with the process of glial scar formation following tissue injury (reactive gliosis). After traumatic injury, astrocytes, microglia and glial progenitor cells around the trauma site are activated and increase in number (Kawano et al., 2012). While surgery promotes astroglial and microglial reactivity, it appears that L-AAA attenuates astrocytic reactivity only without affecting microglial reactivity. Although we did observe increased microglial activation, measured by Iba1 immunoreactivity, following surgery, no effect of L-AAA was observed on microglia reactivity. This result provides additional confirmation of the direct effect of L-AAA on astrocytes and astrocytic specificity of this toxin. In addition, although surgery induces astrogliosis and microgliosis, it doesn't impact depressive-like behaviour in the FST. As FST appears to be less variable than the TST (data not shown), we decided to use the FST to test drugs targeting the NMDA-R/NO signalling pathway.

6.5. Conclusion

In conclusion, the results indicate that normal astrocytic activation follows surgery in the mouse L-AAA administration model, and activation itself is attenuated by administration of the toxin. L-AAA decreases GFAP/S100b immunoreactivity in the PLC and induces depressive-related behaviours without affecting locomotor activity or anxiety-like behaviours. However, it has no effect on the immunoreactivity of the microglial activation marker Iba1 or neuronal marker NeuN. Considering that astrocytes are critical cells for maintaining homeostasis in the brain and that dysfunctional astrocytes in turn affect neuronal function (Liu B., et al., 2017), it is of interest to consider if L-AAA may influence the structural complexity of neurons and dendrites. Indeed a single high dose of L-AAA in rat (20µg/µl, 5 µl) in the mPFC has been reported to produce a complete ablation of astrocytes; a fluorescent halo was observed in the sites where the bilateral injection of L-AAA took place, on which the GFAP staining was completely absent (Lima et al., 2014). L-AAA decreased NeuN immunoreactivity accompanied by reduced neuronal complexity of Golgi-impregnated layer III pyramidal neurons in the mPFC analysed with Neurolucida/NeuroExplorer software (Microbrightfield, Williston, VT, USA). A decrease in neuronal complexity was evident 2 and 6 days following L-AAA administration (Lima et al., 2014) although there were no changes in spine density. Those results suggest that L-AAA, at a higher dose (20µg/µl, 5µl) may exert direct toxic effects to neurons by reducing mature neurons number and affecting their complexity. Our refinement suggests that a low dose of L-AAA (50µg/µl, 1µl) is specific to astrocytes without affecting neurons or microglia numbers. Taken together, investigating the indirect effects of LAAA-induced astroglia toxicity on structural complexity of neurons and dendrites would be an interesting refinement in order to understand the relationship between dysfunctional astrocytes and neurons.

7. Appendix

7.1. Qualitative analysis of the field of distribution of Evan's blue dye following intracortical bolus injection

Results show that the dye injected in the brain spreads 1.7mm from Bregma $\pm$ 0.8mm. 40 slices were visualised with dye. Each slice had a thickness of 0.04mm. The dye spread over a total of 1.6mm and was more concentrated at the area of injection (**Fig 4.6**)

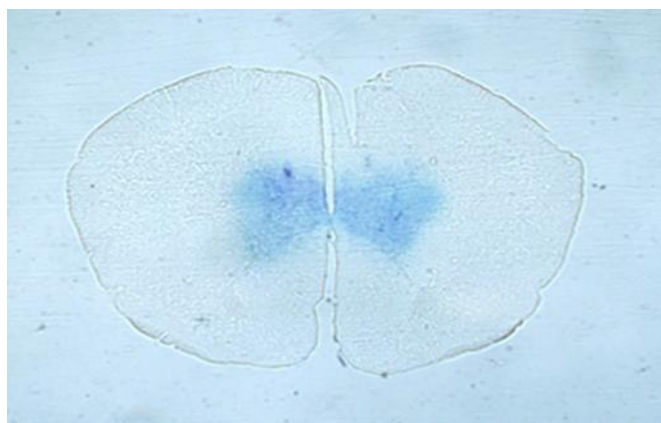


Figure 4.6: Representative image of the distribution of Evan's blue dye following intracortical administration to the mouse

Brains were collected 15 min following intracortical administration using Evan's blue dye to analyse its field of distribution. Evans blue (2% in PBS) was injected bilaterally in the PLC in a similar fashion to L-AAA. The dye spread over a total of 1.6mm and was more concentrated at the site of injection.

7.2. Effect of two doses of L-AAA into the DG on GFAP and DCX immunoreactivity

Reactive astrocytes (increased GFAP immunoreactivity) at the injection site can be seen in **Fig 4.7.A** following administration of PBS. 72 h post L-AAA administration in the DG, GFAP immunoreactivity was reduced but not DCX immunoreactivity. Student's T-test showed an effect of L-AAA on GFAP immunoreactivity in the DG after single bilateral infusion of 50 μ g/ μ l of L-AAA [$T_{(18)} = 3.943$; $P=0.0010$] (**Fig 4.7.D**). However, there was no effect of L-AAA on DCX immunoreactivity [$T_{(18)} = 0.05703$; $P=0.9552$] (**Fig 4.7.E**).

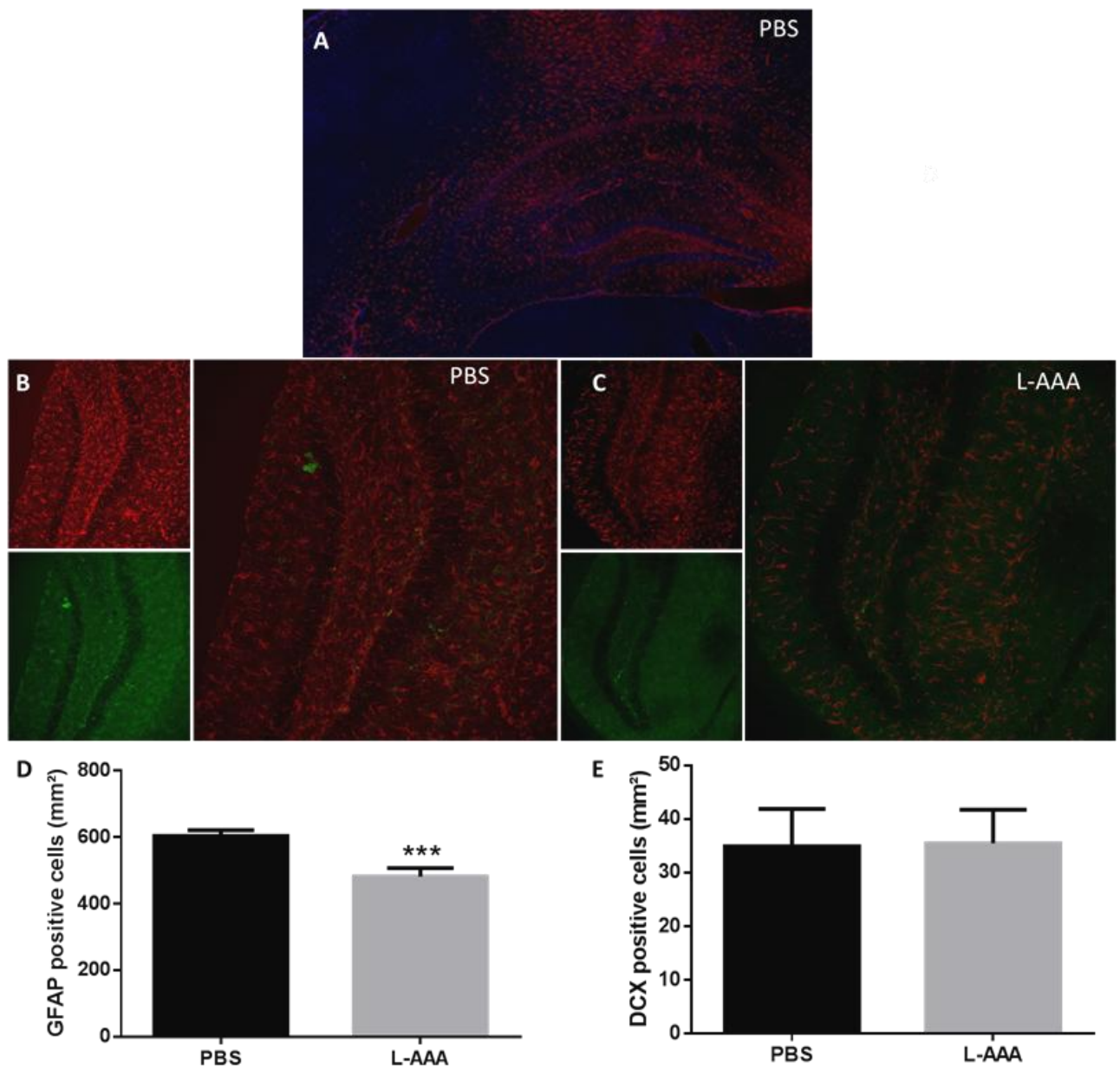


Figure 4.7: Effect of two doses of L-AAA on DCX and GFAP immunoreactivity in the DG

DCX and GFAP immunoreactivity in the DG were analysed 72 h after L-AAA administration. Increased GFAP immunoreactivity is observed at the injection site following PBS administration **A**) GFAP (red), DCX (green) and merged immunoreactivity in the DG are represented in **B**) for PBS control and **C**) for L-AAA-treated mice. Level of GFAP immunoreactivity for both PBS control and L-AAA **D**) and DCX **E**) (PBS; N=10; L-AAA; N=10). Data are expressed as mean $\pm$ SEM *, P<0.05; **, P<0.01, ***, P<0.001 for L-AAA group versus the vehicle-treated control.

Chapter 5

Antidepressant-like properties of ZL006 and associated changes in dendritic spine density following delivery of the astrocytic toxin L-AAA to the PLC in mice

1. Introduction

Previous research from our laboratory demonstrated that targeting glutamate transmission downstream of the NMDA-R using PSD-95/nNOS interaction inhibitors, IC87201 and ZL006, produced antidepressant-like responses in the FST and TST following a single administration in mice. By contrast to the tricyclic antidepressant imipramine, the effects were not observed 1 h following drug administration but were apparent 24 and 72 h later. In addition, prior exposure to the TST or FST was required in order to observe the antidepressant-related activity. Similar delayed and sustained antidepressant-like effects were observed in the TST following administration of the nNOS inhibitor TRIM and NMDA-R antagonist ketamine (Doucet et al., 2013). The antidepressant-related properties of ZL006 have also been reported in Wistar Kyoto rats. Following a second exposure to the FST, ZL006 decreased the immobility in the FST (Sherwin et al., 2017b).

Inhibitors of the NMDA-R/NO signalling pathway acting downstream of the NMDA-R possess a novel mode of action with delayed and sustained antidepressant activity following a single dose compared to conventional antidepressants. As the antidepressant-related properties are only apparent following re-exposure to the FST/TST and 24 h following drug administration, an underlying neurological adaptation, rather than acute pharmacodynamic action may account for their effects. In this regard, antidepressant treatments have been shown to promote changes in neuronal structure, neuronal complexity, spine density, synapse number and integrity in rodents [For reviews, (Duman and Li, 2012; Castrén and Hen, 2013; Duman et al., 2016)]. Effects on neuronal complexity have been mostly studied in animal models of chronic stress. Antidepressant drugs can partially reverse stress-induced dendritic remodelling and synaptic spine reduction [For reviews, (Duman et al., 2016; McEwen et al., 2016)].

Glutamate plays a major role in dendrite and axon development in the CNS. Excessive glutamatergic stimulation promotes neuronal atrophy and reduced complexity with eventual neurodegeneration and cell death (Ernst et al., 2000; Monnerie et al., 2003). In this regard, previous *in vitro* research from our laboratory investigated the effect of uncoupling nNOS from the NMDA-R with PSD-95/nNOS inhibitors IC87201 and ZL006 on NMDA-glycine-induced neuronal atrophy *in vitro*. Under these experimental conditions, ketamine (10nM) and TRIM (100nM) counteracted the NMDA/glycine-induced reduction in neurite outgrowth. Treatment with the small-molecule inhibitors of the PSD-95/nNOS interface IC87201 (10

and 100nM) and ZL006 (10 and 100nM) attenuated NMDA/glycine-induced decreases in neurite outgrowth (M.V. Doucet et al., 2015). As previously mentioned, inhibitors of the PSD-95/nNOS interaction require a prior exposure to the TST or FST in order to produce antidepressant-related activity. Therefore, a modified FST protocol in the mouse will be employed in this investigation with a first exposure to the FST (baseline ‘stress’) and a re-exposure to the FST 24 h later. It has been demonstrated that when mice are re-exposed to the FST, animals show an increase in immobility in the FST compared to the first exposure (stress response) and ZL006 and IC87201 are able to block the increase in immobility observed on second exposure (Doucet et al., 2013). Other research has demonstrated that ZL006 was able to reverse the neuronal atrophy of cultured cortical neurons induced by high concentrations of glutamate (Zhou et al., 2010) suggesting that targeting the NMDA-R/PSD-95/nNOS interaction downstream of the NMDA-R attenuates atrophic effects by preventing neurite shrinkage in response to excessive glutamatergic stimulation. However, there are limitations to measuring neuronal complexity *in vitro*. Sholl analysis *in vitro* can only be performed on immature neurons in order to visualise individual neurons. As mature neurons have higher sensitivity to glutamate than immature neurons (Monnerie et al., 2003), it is of interest to assess the effect of PSD-95/nNOS interaction inhibitors in mature neurons. It is also of importance to investigate the impact of pathway inhibitors on other elements relating to neuronal plasticity such as spine density on mature neurons *in vitro* by using transfected neurons described in chapter 3 or *in vivo* in the rodent brain. Spines are dynamic, changing shape and form between seconds and hours (De Roo et al., 2008). These are categorised into thin spines which are highly plastic and transient (Bourne and Harris, 2007), mushroom which are stable, mature, and persistent (Yasumatsu et al., 2008) and stubby which are more persistent and stable than thin spines (Sebastian et al., 2013).

There is emerging evidence linking stress-related changes in dendritic spine density homeostasis, dendrite arborisation, and neuronal complexity with the pathophysiology of stress-related psychiatric disorders such as depression (Sato, 2013; Duman and Duman, 2015). Exposure to stressful life events is a major risk factor associated with the onset of depression (Kessler, 1997). Animal studies have revealed that chronic stress is associated with neuronal atrophy and dendritic spine loss in the PFC and hippocampus [For review, (Popoli et al., 2012; McEwen and Morrison, 2013; Duman and Duman, 2015)].

Taking into consideration that post-mortem studies revealed that the astrocytic marker GFAP is decreased in brains of depressed patients [For review (Rajkowska et al. 2013)] and that

astrocytes play a major role in glutamate regulation and provision of trophic support to neurons (Barres, 2008; Allen, 2013), a new animal model of depression based on astrocytic ablation has recently emerged. The astrocytic toxin L-AAA previously discussed in chapters 3 and 4 has received particular attention in recent years as an effective method of inducing toxicity in astrocytic cells. *In vitro* results from chapter 3 demonstrated that CM taken from L-AAA-treated astrocytes promotes neuronal atrophy in immature primary cortical neurons and reduces synapse formation and spine density in mature neurons. In chapter 4, results indicate that a single dose of L-AAA administered in the PLC of mice (50µg/ul; 1µl) was sufficient to decrease GFAP and S100b immunoreactivity (astrocytic markers) and induce depressive-like behaviours as previously reported (Banasr and Duman, 2008; Lee et al., 2013; Liu F., et al., 2017). In mice, two consecutive doses of L-AAA in the PLC (100µg/µl, 0.6µl, 2 injections) reduced levels of glutamate/glutamine in the PFC 5 days after the second injection. These results support the hypothesis that astrocyte loss and glutamate dysregulation contribute to the pathophysiology underlying depressive behaviour (Banasr and Duman, 2008; Lee et al., 2013).

A recent study using golgi staining in rats demonstrated that L-AAA-induced astrocytic dysfunction promotes dendritic atrophy but did not affect spine density to the PFC following L-AAA administration (20µg/µl, 5µl, 1 injection) (Lima et al., 2014). 2 days post L-AAA administration; L-AAA didn't affect apical but decreased basal dendritic length. After 6 days, L-AAA decreased both apical and basal dendritic length. However, L-AAA didn't affect the number of apical, basal dendrites or apical, basal dendritic spine density 2 or 6 days post administration. These results suggest that neuronal atrophy observed in this study is a consequence of the L-AAA-induced astrocyte loss. As this study used a high dose of L-AAA in rat and placement of a guide cannula which can trigger activation of astroglia and microglia, it is of interest to assess the effect of a lower dose of L-AAA (50µg/µl, 1µl, 1 injection) in mice injected via microcapillary (as described in chapter 4) on neuronal complexity and spine density. In addition, it would be of interest to investigate *in vivo* if inhibitors of the PSD-95/nNOS interaction can reverse L-AAA-induced depressive-like behaviour and assess their effect on neuronal complexity and dendritic spine density following L-AAA-induced astrocytic ablation in the PLC.

As previously discussed, PSD-95/nNOS may influence stress-induced adaptive changes in neural transmission and plasticity. Taking into consideration that astrocytic ablation with L-AAA administration impairs glutamate/glutamine levels in the PFC in mice (Lee et al. 2013) and decreases neuronal complexity in the mPFC in rat (Lima et al. 2014) and that inhibitors of PSD-95/nNOS interaction modulate the glutamate pathway, the effects of inhibiting PSD-95/nNOS interaction on L-AAA-induced neuronal atrophy *in vitro* were investigated in chapter 3. This research demonstrated that *in vitro*, CM from astrocytes treated with L-AAA (0.5mM) induced neuronal atrophy and synapse loss which were attenuated by treating the neurons with ZL006 and IC87201. As we obtained more robust effects on neuronal structure following ZL006 than following IC87201 treatment *in vitro* in chapter 3, here in this chapter we will examine the effect of ZL006 rather than IC87201 on dendritic spine density *in vivo*.

2. Aims and objectives

Firstly, this study aims to assess the effect of ZL006 administration in naive mice on neuronal complexity and dendritic spines in the PLC. The anti-depressant-related properties of ZL006 in the FST and for comparison purposes, TRIM are investigated in addition to assessing neuronal complexity and dendritic spine formation in the PLC.

Second, the effect of L-AAA delivery in the PLC is assessed on depressive-like behaviour in the FST and associated changes in dendritic spine density and number of primary neurites.

Finally, this investigation examines the effect of ZL006 on immobility in the mouse FST and associated changes in dendritic spine density and number of primary neurites following L-AAA-induced astrocytic toxicity in the PLC.

3. Methods

3.1. Drug administration

Drugs were prepared freshly and injected intra-peritoneally in an injection volume of 10ml/kg. Doses were as follows: TRIM 50mg/kg and ZL006 10mg/kg in 0.9% saline and vehicle (0.9% saline).

3.2. Stereotactic delivery of L-AAA in the PLC

Male C57Bl6/J 9-12 weeks old (BioResources, Trinity College Dublin) were anaesthetised by IP injection using avertin (1.25g 2,2,2, tribromoethanol; 2.5ml tertiary amyl alcohol; 100ml distilled water) and secured in a stereotactic frame. L-AAA (Sigma Aldrich) (50µg/µl; 1µl; 0.2µl/min) or PBS was administered bilaterally into the pre limbic cortex (PLC; 1.7mm anteroposterior, -0.4mm dorsolateral and depth -2.5mm from Bregma).

3.3. Behaviour in the mouse FST

All animals were handled daily for 5 days prior to surgery and behavioural testing. Mice were firstly exposed to the FST and subsequently injected with ZL006, TRIM or vehicle; 24 h later animals were re-exposed to the FST to assess the antidepressant-like effect of the drugs. Non-FST exposed groups were included for comparison purposes (**Fig 5.1-5**).

3.4. Golgi-cox staining of neurites and dendritic spines

Golgi-cox staining was performed using the super Golgi Kit designed for the labelling of dendritic branches and spines of neurons, based on the principle of Golgi-Cox impregnation (Bioenno Tech, Irvine, CA, USA) (for details refer to chapter 2 material and methods). Mice were transcardially perfused for 3 min with saline 0.9% solution. Brains were rinsed and incubated for 10 days in solution A (Impregnation solution) and transferred into solution B (Post-impregnation buffer: 30g of reagent B in 90ml dH₂O) for 1 day, renewed and kept until use at 4°C. Using a vibratome, 150µm coronal sections were collected into solution B (Collection solution: 30g of reagent B in 500ml dH₂O) and mounted on gelatine subbed slides. Due to time constraints, lack of appropriate software and limitations of golgi-cox staining, the number of primary neurites only was analysed as a measure of neuronal complexity in this investigation. This measure is mostly used *in vitro* as a measure of neuronal complexity (M.V. Doucet et al., 2015; O'Neill et al., 2016). Image stacks of the regions of interest were acquired in the PLC at a magnification 200x (20x objective) with an

Olympus IX81-long focal length fluorescent microscope to analyse the number of primary neurites emerging from the soma. Z-stacks were acquired for spine analysis at a magnification 600x (60x objective). 7 apical and 5 basal dendritic segments of 30µm were acquired per neuron and from 5 neurons per animal (3-4 mice per group). Dendritic segments were selected only 1) for non-truncated dendrites, 2) with a dark and uniform staining along all projections and 3) if they were relatively isolated from neighbouring stained neurons. Spine quantification was performed using Neuron Studio software and spine morphology was classified. Individual spine length and width were measured and classified according to one of the following subtypes: stubby (no neck), mushroom (head diameter: 4µm and neck length <2µm), thin (head diameter smaller than the neck length: >2µm). All values were calculated by averaging the number of spines, divided by the dendritic length of the sample.

4. Study design

4.1. Effect of ZL006 on dendritic spine density and number of primary neurites

To assess the effect of ZL006 on dendritic spine density and neuronal complexity, C57BL6/J mice received an IP injection of vehicle or ZL006 (10mg/kg). 24 h following the IP injection, the animals were euthanised and brains were collected for golgi-cox staining.

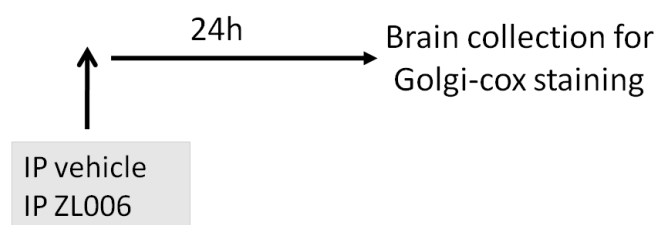


Figure 5.1: Effect of ZL006 on dendritic spine density and number of primary neurites

4.2. Effect of FST on dendritic spine density and number of primary neurites

To assess the effect of swim stress (FST exposure) on dendritic spine density and neuronal complexity, C57BL6/J mice were firstly exposed to the FST, subsequently received an IP injection of vehicle and were re-exposed to the FST 24 h later. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox staining. Another group received an IP injection without FST exposure and a third group remained undisturbed (home cage group). 24 h following the IP injection, the animals were euthanised and brains were collected for golgi-cox staining.

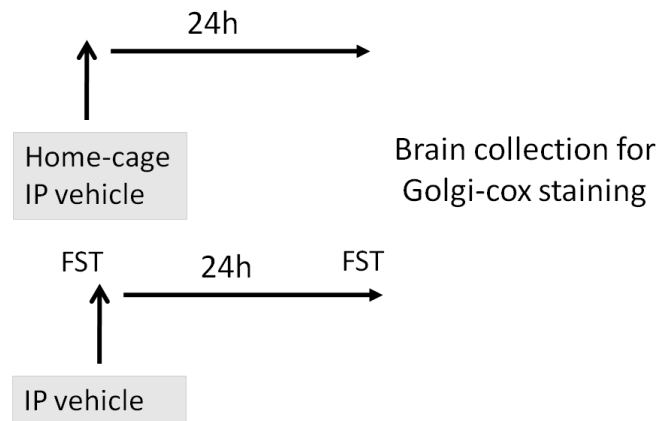


Figure 5.2: Effect of swim stress on dendritic spine density and number of primary neurites

4.3. Effect of ZL006 and TRIM in the mouse FST and associated changes in dendritic spine density and number of primary neurites

To assess the effect of ZL006 or TRIM on dendritic spine density and neuronal complexity in FST exposed mice, C57BL6/J mice were exposed to the FST, subsequently received an IP injection of vehicle, TRIM (50mg/kg) or ZL006 (10mg/kg) and were re-exposed to the FST 24 h after. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox staining.

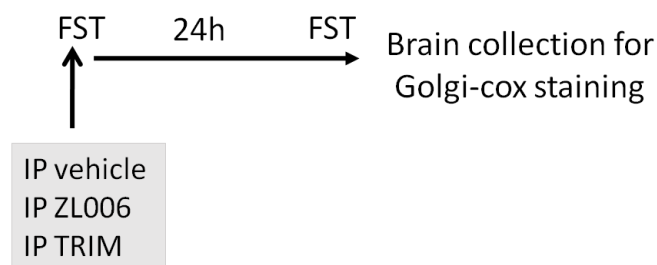


Figure 5.3: Effect of ZL006 and TRIM in the mouse FST and associated changes in dendritic spine density and number of primary neurites

4.4. Effect of stereotactic delivery of L-AAA to the PLC on dendritic spine density and number of primary neurites

To assess the effect of L-AAA on dendritic spine density and neuronal complexity, L-AAA (50µg/µl; 1µl) or PBS was administered to the PLC. 72 h following L-AAA administration, the animals were euthanised and brains were collected for golgi-cox staining.

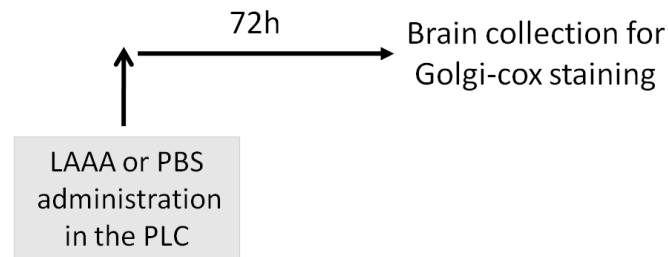


Figure 5.4: Effect of stereotactic delivery of L-AAA to the PLC on dendritic spine density and number of primary neurites

4.5. Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

L-AAA (50µg/µl; 1µl) or PBS was administered to the PLC, 48 h later mice were exposed to the FST and subsequently received an IP injection of vehicle or ZL006 (10mg/kg). Mice were re-exposed to the FST 24 h later. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox staining

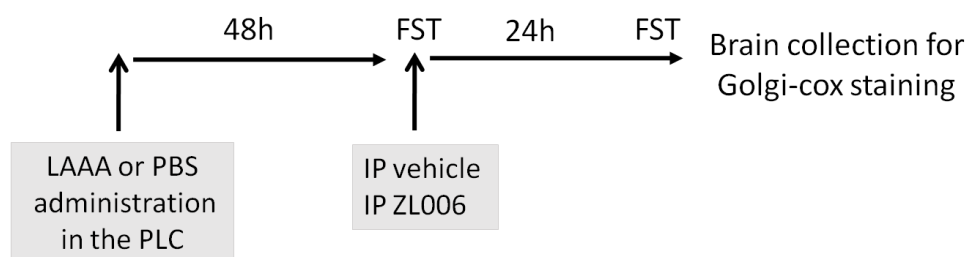


Figure 5.5: Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

5. Statistical analysis

Data are expressed as group mean with SEM and were analysed using unpaired Student's *t*-tests or analysis of variance (ANOVA). If any statistically significant change was found following one or two factor ANOVA, *post-hoc* comparisons were performed using a Fisher's LSD test. Data were deemed significant when $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

6. Results

6.1. Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC in mice

A two-way ANOVA of spine density on apical dendrites showed no effect of ZL006 [$F_{(1,84)}=0.48$, $P=0.49$], an effect of spine subtype [$F_{(2,84)}=20$ $P<0.001$] but no ZL006 x spine subtype interaction [$F_{(2,84)}=0.078$, $P=0.92$]. A two-way ANOVA of spine density on basal dendrites showed no effect of ZL006 [$F_{(1,84)}=2.2$, $P=0.14$], an effect of spine subtype [$F_{(2,84)}=7.08$, $P=0.0014$] but no ZL006 x spine subtype interaction [$F_{(2,84)}=0.33$, $P=0.71$](**Fig 5.6.E,F**). Student's T-test showed no effect of ZL006 on the number of primary neurites 24 h post administration [$T_{(28)}=0.09$, $P=0.92$] when compared to controls (**Fig 5.6.G**).

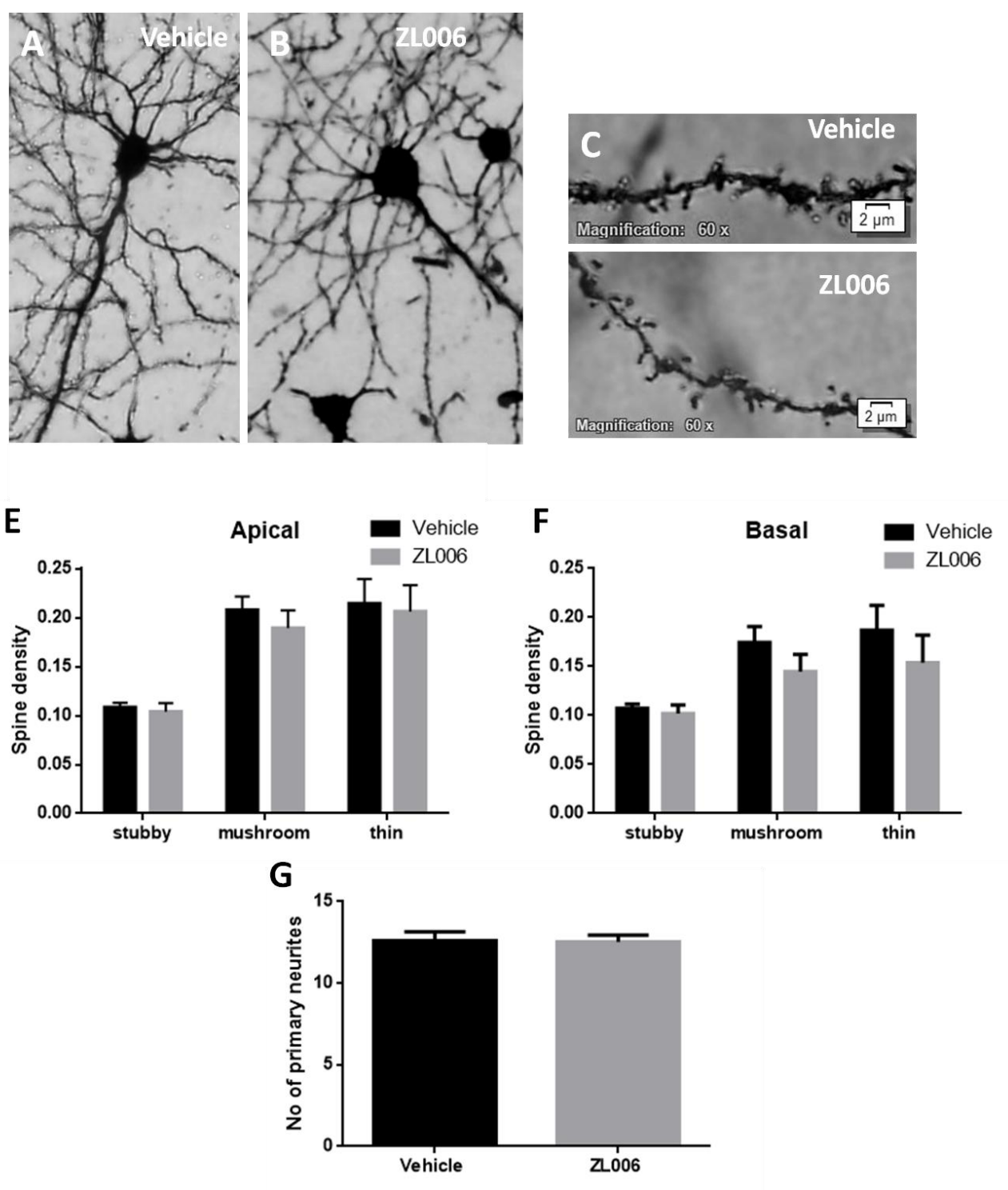


Figure 5.6: Effect of ZL006 administration on dendritic spine density and number of primary neurites in the PLC in mice

C57BL6/J mice received an IP injection of ZL006 (10mg/kg) or vehicle injection. 24 h following the IP injection, the animals were euthanised and brains were collected for golgi-cox staining. Representative picture for vehicle (**A,C**), ZL006 (**B,D**). ZL006 administration didn't increase spine density on apical (**E**) or basal (**F**) dendrites. ZL006 did not affect the number of primary neurites 24 h post administration (**G**) Data are expressed as mean $\pm$ SEM, (Vehicle= 15 neurons; ZL006= 15 neurons, 3 mice per group), *** P <0.001 ** P <0.01 and * P <0.05 vs. vehicle treated controls.

6.2. Effect of FST exposure on dendritic spine density and number of primary neurites in the PLC in mice

A two-way ANOVA of spine density on apical dendrites showed an effect of FST [$F_{(2,153)} = 4.42$, $P = 0.01$], an effect of spine subtype [$F_{(2,153)} = 35.37$, $P < 0.001$] but no FST x spine subtype interaction [$F_{(4,153)} = 0.85$, $P = 0.49$]. A two-way ANOVA of spine density on basal dendrites showed an effect of FST [$F_{(2,150)} = 9.33$, $P < 0.001$], an effect of spine subtype [$F_{(2,150)} = 11.65$, $P < 0.001$] but no FST x spine subtype interaction [$F_{(4,150)} = 0.66$, $P = 0.61$]. Post hoc comparisons revealed that mice exposed to the FST had reduced thin spine density on apical dendrites ($P < 0.05$) compared to the home cage group and lower mushroom spine density ($P < 0.05$) and thin spine density ($P < 0.01$) on basal dendrites when compared to the IP injection group (**Fig 5.7.G,H**). Animals given an IP injection had higher mushroom and thin spine density [$P < 0.05$] on basal dendrites when compared to the home cage group. One way ANOVA of the number of primary neurites showed an effect of FST [$F_{(2,50)} = 10.09$, $P < 0.001$]. Post hoc comparisons revealed that mice exposed to the FST had a lower number of primary neurites compared to their home cage counterparts [$P < 0.01$] (**Fig 5.7.I**).

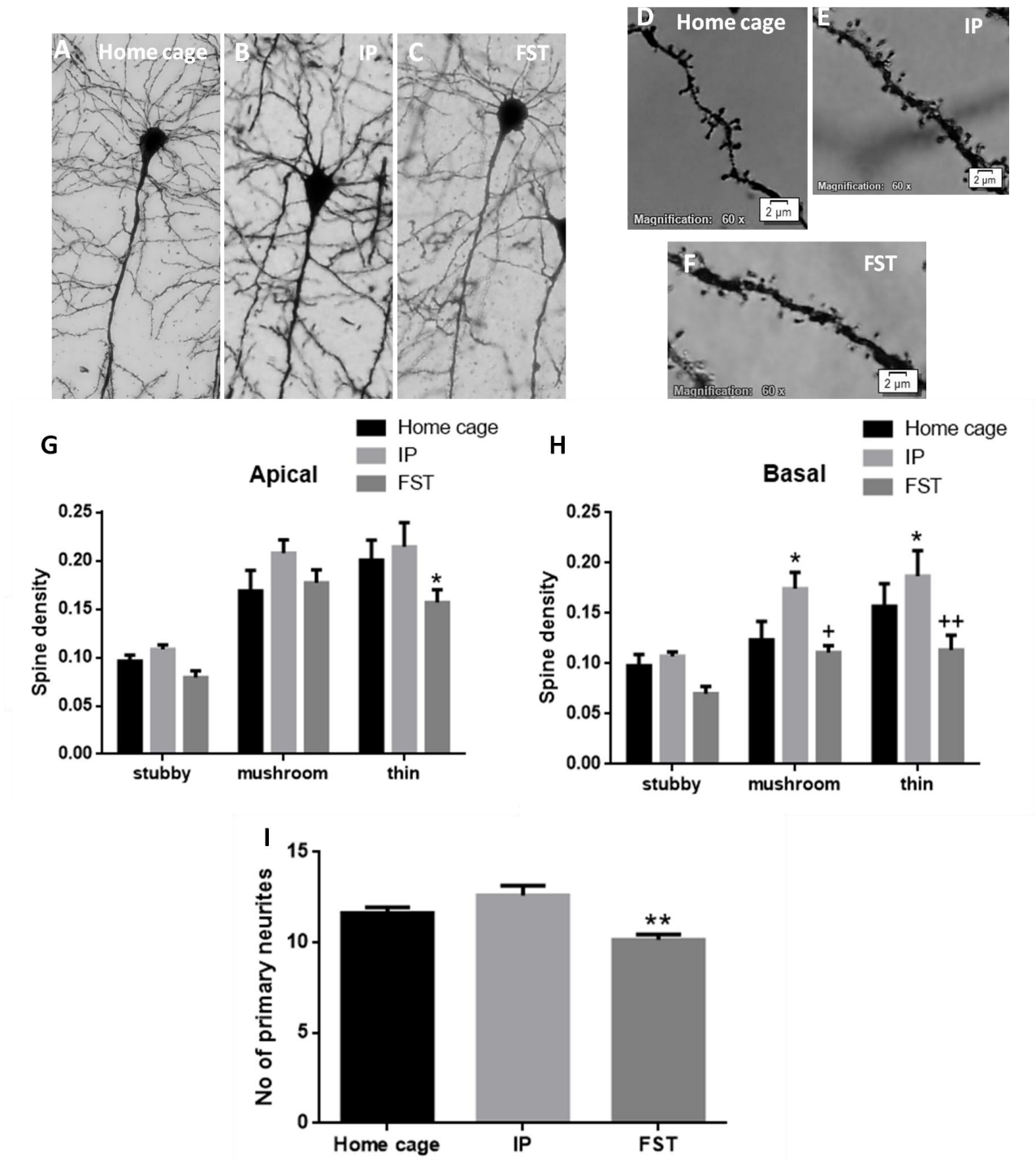


Figure 5.7: Effect of FST exposure on dendritic spine density and number of primary neurites in the PLC in mice

C57BL6/J mice were firstly exposed to the FST, subsequently received an IP injection of vehicle and were re-exposed to the FST 24 h later. Another group received an IP injection without FST exposure and a third group remained undisturbed (home cage group). 24 h following the IP injection, the animals were euthanised and brains were collected for golgi-cox staining. Representative picture for home cage group (A,D), IP injection group (B,E), FST group (C,F). FST decreased thin spine density on apical dendrites (G). IP increased mushroom and thin spines and FST decreased mushroom and thin spines on basal dendrites (H). FST but not IP reduced the number of primary neurites (I) Data are expressed as mean $\pm$ SEM, (Home cage= 20 neurons; IP= 15 neurons; FST= 18 neurons, 3-4 mice per group), *** P <0.001 ** P <0.01 and * P <0.05 vs. home cage counterparts, + P <0.05 ++ P <0.01 vs IP counterparts.

6.3. Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC following FST exposure in mice

A two-way repeated measure ANOVA of immobility in the FST showed no effect of ZL006 [$F_{(1,19)} = 0.9$, $P = 0.35$], an effect of time [$F_{(1,19)} = 14.62$, $P = 0.0011$] and a ZL006 x time interaction [$F_{(1,19)} = 8.86$, $P = 0.007$]. Post hoc comparisons revealed that ZL006 decreased immobility in the FST 24 h after administration when compared to vehicle-treated controls ($P < 0.05$) (**Fig 5.8.H**).

A two-way ANOVA of spine density on apical dendrites showed an effect of ZL006 [$F_{(1,108)} = 4.45$, $P = 0.037$], an effect of spine subtype [$F_{(2,108)} = 46.23$, $P < 0.001$] and a ZL006 x spine subtype interaction [$F_{(2,108)} = 3.36$, $P = 0.029$]. A two-way ANOVA of spine density on basal dendrites showed an effect of ZL006 [$F_{(1,108)} = 7.97$, $P = 0.005$], an effect of spine subtype [$F_{(2,108)} = 16.68$, $P < 0.001$] but no ZL006 x spine subtype interaction [$F_{(2,108)} = 1.68$, $P = 0.19$]. Post hoc comparisons revealed that ZL006 increased thin spine density on apical dendrites compared to vehicle-treated controls ($P < 0.001$) and mushroom and thin spine density on basal dendrites ($P < 0.05$) (**Fig 5.8.E**). Student's T-test showed no effect of ZL006 on the number of primary neurites 24 h post administration [$T_{(37)} = 0.73$, $P = 0.46$] when compared to vehicle-treated controls (**Fig 5.8.G**).

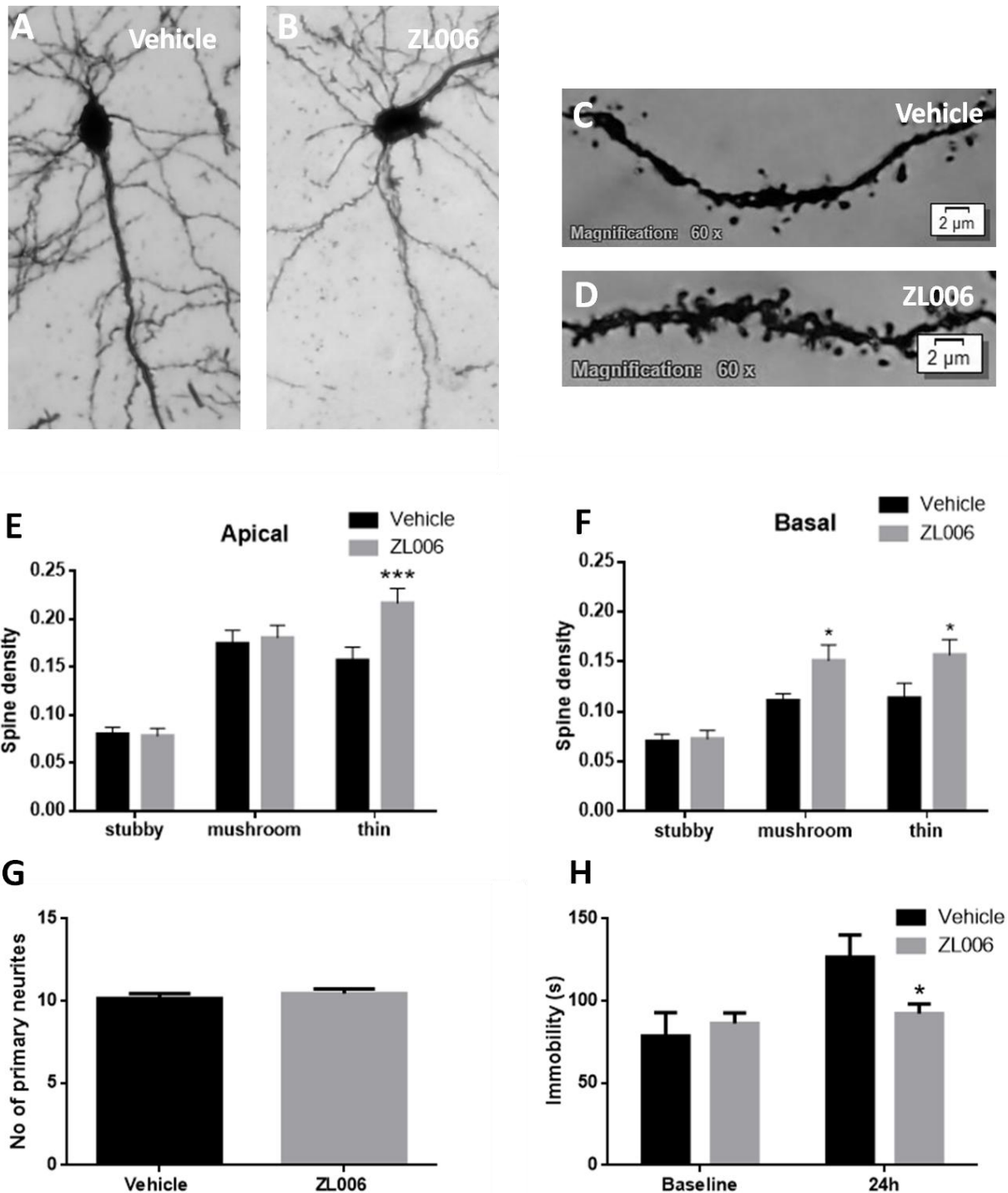


Figure 5.8: Effect of ZL006 administration on dendritic spine density and number of primary neurites in the PLC following FST exposure in mice

C57BL6/J mice were exposed to the FST, subsequently received an IP injection of vehicle or ZL006 (10mg/kg) and were re-exposed to the FST 24 h after. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox staining. Representative picture for vehicle FST group (A,C), ZL006 FST group (B,D). ZL006 increased thin spine density on apical (E) but didn't have any effect on basal dendrites (F). ZL006 did not affect the number of primary neurites 24 h post administration (G) Data are expressed as mean $\pm$ SEM, Vehicle= 18 neurons; ZL006= 20 neurons, 4 mice per group). ZL006 decreased immobility in the FST 24 h post administration (H) (Vehicle= 11 mice, ZL006= 10 mice) *** P <0.001 ** P <0.01 and * P <0.05 vs. vehicle treated controls.

6.4. Effect of TRIM on dendritic spine density and number of primary neurites following FST exposure in mice

A two-way ANOVA of immobility in the FST showed no effect of TRIM [$F_{(1,18)} = 4.04$, $P = 0.059$], no effect of time [$F_{(1,18)} = 0.29$, $P = 0.59$] but a TRIM x time interaction [$F_{(1,18)} = 8.08$, $P = 0.0108$]. Post hoc comparisons revealed that TRIM decreased immobility in the FST 24 h after administration when compared to vehicle-treated controls [$P < 0.01$] (**Fig 9.H**).

A two-way ANOVA of spine density on apical dendrites showed no effect of TRIM [$F_{(1,108)} = 1.31$, $P = 0.25$], an effect of spine subtype [$F_{(2,108)} = 31.4$, $P < 0.001$] and no TRIM x spine subtype interaction [$F_{(2,108)} = 0.11$, $P = 0.89$] (**Fig 9.E**). A two-way ANOVA of spine density on basal dendrites showed an effect of TRIM [$F_{(1,108)} = 0.15$, $P = 0.69$], an effect of spine subtype [$F_{(2,108)} = 14.26$, $P < 0.001$] but no TRIM x spine subtype interaction [$F_{(2,108)} = 0.64$, $P = 0.52$] (**Fig 5.F**). Post hoc comparisons failed to reveal an effect of TRIM when compared to vehicle-treated controls.

Student's T-test showed that TRIM increased the number of primary neurites 24 h post administration [$T_{(38)} = 2.69$, $P = 0.0105$] when compared to vehicle-treated controls (**Fig 5.G**).

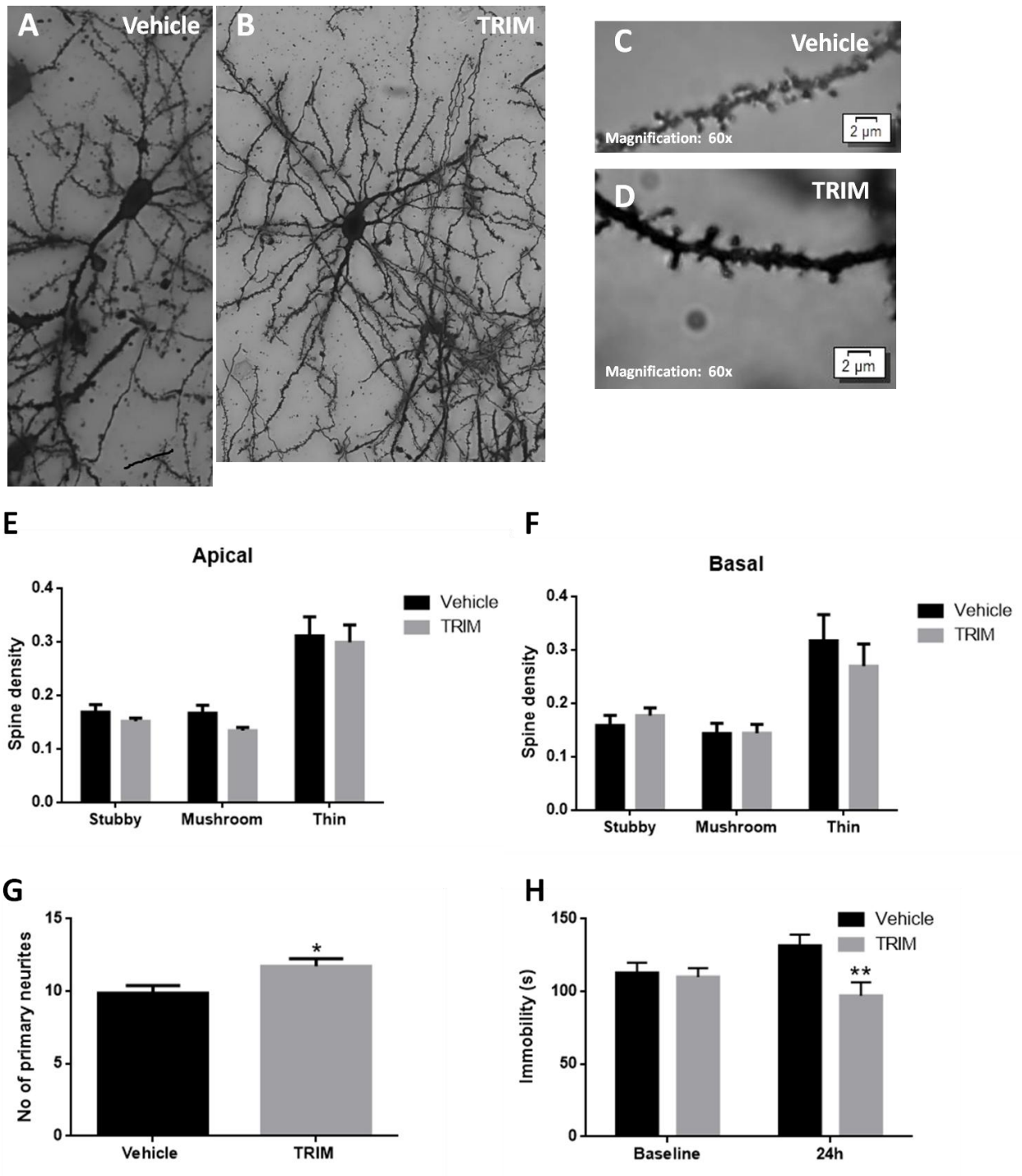


Figure 5.9: Effect of TRIM administration on dendritic spine density and number of primary neurites in the PLC following FST exposure in mice

C57BL6/J mice were exposed to the FST, subsequently received an IP injection of vehicle or TRIM (50mg/kg) and were re-exposed to the FST 24 h after. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox. Representative picture for vehicle FST group (A.C), TRIM FST group (B.D). TRIM did not have any effect on apical dendrites (E) but had a main effect on basal dendrites (F). TRIM increased the number of primary neurites 24 h post administration (G) Data are expressed as mean $\pm$ SEM, (Vehicle= 15 neurons; TRIM= 18 neurons, 3-4 mice). TRIM decreased immobility in the FST 24 h post administration (H) (Vehicle= 10 mice, TRIM= 10 mice), *** P <0.001 ** P <0.01 and * P <0.05 vs. vehicle treated controls.

6.5. L-AAA delivery to the PLC reduces dendritic spine density and the number of primary neurites in the PLC of mice

A two-way ANOVA of spine density on apical dendrites showed an effect of L-AAA [$F_{(1,84)} = 8.99$, $P = 0.003$], an effect of spine subtype [$F_{(2,84)} = 55.96$, $P < 0.001$] and a L-AAA x spine subtype interaction [$F_{(2,84)} = 5.364$, $P = 0.006$]. A two-way ANOVA of spine density on basal dendrites showed no effect of L-AAA [$F_{(1,84)} = 0.69$, $P = 0.4$], an effect of spine subtype [$F_{(2,84)} = 25.22$, $P < 0.001$] and a L-AAA x spine subtype interaction [$F_{(2,84)} = 3.16$, $P = 0.04$]. Post hoc comparisons revealed that mice treated with L-AAA (50 $\mu\text{g}/\mu\text{l}$; 1 μl) had lower mushroom spine density on apical dendrites ($P < 0.001$) and lower mushroom spine density ($P < 0.05$) on basal dendrites when compared to PBS controls (**Fig 5.10.E,F**). Student's T-test showed no effect of L-AAA on the number of primary neurites 72 h post administration [$T_{(28)} = 0.17$, $P = 0.86$] when compared to PBS controls (**Fig 5.10.G**).

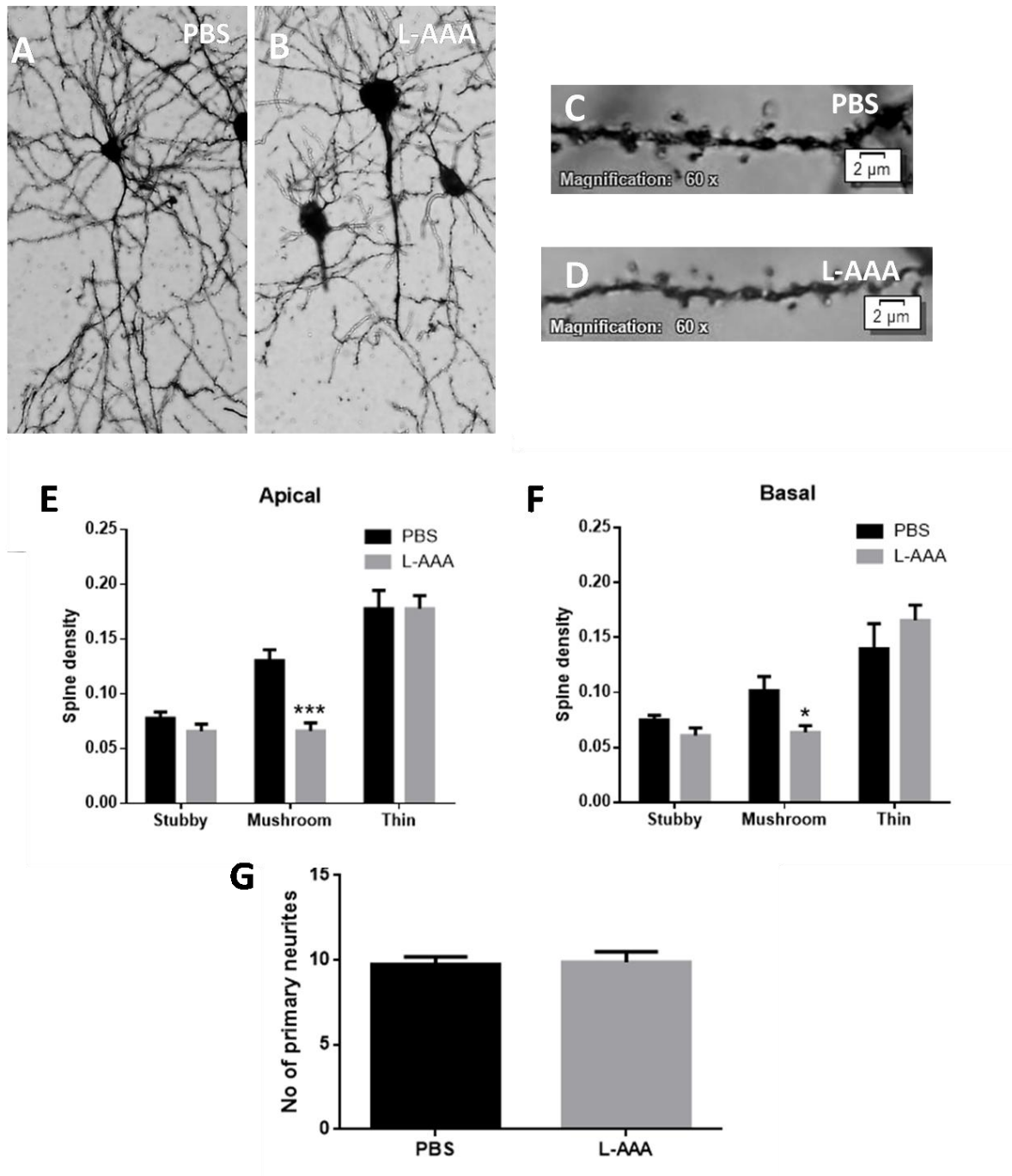


Figure 5.10: Effect of L-AAA delivery to the PLC on dendritic spine density and number of primary neurites in the PLC of mice

C57BL6/J mice received one bilateral injection of L-AAA (50 μ g/ μ l; 1 μ l) into the PLC. 72 h after L-AAA administration, the animals were euthanised and brains were collected for golgi-cox staining. Representative picture for PBS control group (A,C) and L-AAA (B,D). L-AAA decreased mushroom spine density on apical dendrites (E) and basal dendrites (F) but did not affect the number of primary neurites 72 h post administration (G) Data are expressed as mean $\pm$ SEM, (PBS= 15 neurons; L-AAA= 15 neurons, 3 mice per group), ***P<0.001 **P<0.01 and *P<0.05 vs. PBS controls.

6.6. Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

6.6.1. Effect of FST exposure in L-AAA-treated mice on dendritic spine density and number of primary neurites in the PLC

A two-way ANOVA of spine density on apical dendrites showed an effect of FST [$F_{(1,96)} = 30.56$, $P < 0.001$], an effect of spine subtype [$F_{(2,96)} = 33.04$, $P < 0.001$] and no FST x spine subtype interaction [$F_{(2,96)} = 0.48$, $P = 0.61$] (**Fig 11.A**). Post hoc comparisons revealed that FST combined with L-AAA treatment increased stubby ($P < 0.001$), mushroom ($P < 0.01$) and thin ($P < 0.001$) spine density on apical dendrites when compared to their non-FST counterparts. A two-way ANOVA of spine density on basal dendrites showed an effect of FST [$F_{(1,96)} = 27.72$, $P < 0.001$], an effect of spine subtype [$F_{(2,96)} = 18.7$, $P < 0.001$] but no FST x spine subtype interaction [$F_{(2,96)} = 1.44$, $P = 0.24$] (**Fig 11.B**). Post hoc comparisons revealed that FST combined with L-AAA increased stubby ($P < 0.001$), mushroom ($P < 0.05$) and thin ($P < 0.05$) spine density on basal dendrites when compared to their non-FST counterparts. A two-way ANOVA of spine density on apical dendrites showed an effect of FST [$F_{(1,84)} = 9.29$, $P = 0.003$], an effect of spine subtype [$F_{(2,84)} = 9.74$, $P < 0.001$] and no FST x spine subtype interaction [$F_{(2,84)} = 0.95$, $P = 0.38$]. Post hoc comparisons revealed that FST combined with PBS treatment increased stubby ($P < 0.01$) spine density on apical dendrites when compared to their non-FST counterparts. A two-way ANOVA of spine density on basal dendrites showed an effect of FST [$F_{(1,84)} = 4.84$, $P = 0.03$], an effect of spine subtype [$F_{(2,84)} = 3.95$, $P = 0.02$] but no FST x spine subtype interaction [$F_{(2,84)} = 2.21$, $P = 0.11$]. Post hoc comparisons revealed that FST combined with PBS increased stubby ($P < 0.01$) spine density on basal dendrites when compared to their non-FST counterparts (data not shown).

Student's T-test showed no effect of the FST on the number of primary neurites 72 h post administration [$T_{(28)} = 1.06$, $P = 0.29$] when compared to their non-FST counterparts (**Fig 5.11.C**).

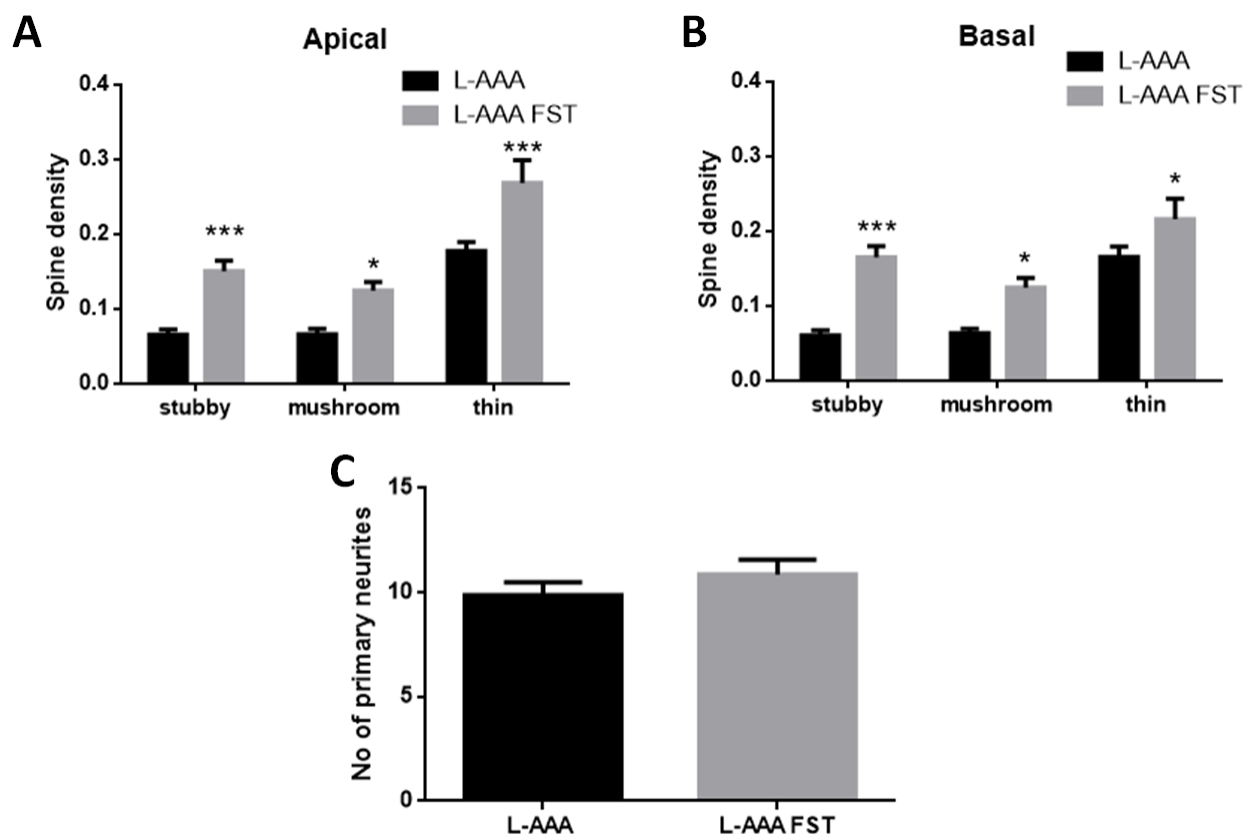


Figure 5.11: Effect of FST exposure in L-AAA-treated mice on dendritic spine density and number of primary neurites in the PLC

C57BL6/J mice received one bilateral injection of L-AAA into the PLC (50µg/µl; 1µl) and 48 h later were exposed or not to the FST. The group exposed to the FST received an IP injection of saline and was re-exposed to the FST 24 h later. 72 h after L-AAA administration, the animals were euthanised and brains were collected for golgi-cox staining. FST combined with L-AAA increased stubby, mushroom and thin spine densities on apical dendrites and stubby and mushroom spine densities on basal dendrites (**A,B**). L-AAA didn't affect the number of primary neurites (**C**). Data are expressed as mean ± SEM, (L-AAA= 15 neurons; L-AAA-FST= 19 neurons, 3-4 mice) ***P<0.001 **P<0.01 and *P<0.05 vs the non-FST counterparts.

6.6.2. Effect of ZL006 in the FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

Student's T-test showed that L-AAA increased immobility in the FST at the baseline (before treatment) 48 h after L-AAA administration [$T_{(36)} = 4.32$, $P < 0.001$] when compared to PBS controls (**Fig 12.E left**). As L-AAA-treated mice exhibited an increase of immobility in the FST compared to PBS treated controls, animals treated with L-AAA were subdivided into two subgroups of equal immobility and randomly injected with either vehicle or ZL006. Mice were re-exposed to the FST 24 h after. Student's T-test showed a reduction in immobility in the ZL006 group in L-AAA-treated animals [$T_{(16)} = 2.64$, $P = 0.017$] (**Fig 12.E right**).

PBS treated mice were also subdivided into two subgroups of equal immobility and randomly injected with either vehicle or ZL006. Mice were re-exposed to the FST 24 h after. Student's T-test showed no effect of ZL006 on immobility in the PBS group in the FST 24 h after ZL006 treatment [$T_{(18)} = 1.41$, $P = 0.17$] (data not shown).

Following L-AAA administration, a two-way ANOVA of spine density on apical dendrites showed no effect of ZL006 [$F_{(1,108)} = 0.12$, $P = 0.72$], an effect of spine subtype [$F_{(2,108)} = 17.77$, $P < 0.001$] and a ZL006 x spine subtype interaction [$F_{(2,108)} = 3.41$, $P = 0.036$] (**Fig 12.F**). A two-way ANOVA of spine density on basal dendrites showed no effect of ZL006 [$F_{(1,108)} = 2.85$, $P = 0.09$], an effect of spine subtype [$F_{(2,108)} = 6.31$, $P = 0.026$] with no ZL006 x spine subtype interaction [$F_{(2,108)} = 1.68$, $P = 0.19$] (**Fig 12.G**). Post hoc comparisons revealed a reduced number of thin spines in apical dendrites following ZL006 treatment when compared to vehicle treated controls.

After PBS administration, a two-way ANOVA of spine density on apical dendrites showed no effect of ZL006 [$F_{(1,81)} = 0.038$, $P = 0.84$], an effect of spine subtype [$F_{(2,81)} = 16.92$, $P < 0.001$] with no ZL006 x spine subtype interaction [$F_{(2,81)} = 3.08$, $P = 0.051$] (**Fig 12.H**). A two-way ANOVA of spine density on basal dendrites showed no effect of ZL006 [$F_{(1,81)} = 0.0004$, $P = 0.98$], an effect of spine subtype [$F_{(2,81)} = 3.28$, $P = 0.04$] and no ZL006 x spine subtype interaction [$F_{(2,81)} = 0.4$, $P = 0.667$] (**Fig 12.I**).

Student's T-test showed no effect of ZL006 on the number of primary neurites 24 h post administration in mice treated with L-AAA [$T_{(28)} = 0.15$, $P = 0.87$] (**Fig 5.12.J**). Student's T-test showed no effect of ZL006 on the number of primary neurites 24 h post administration in mice treated with PBS [$T_{(28)} = 0.10$, $P = 0.91$] (**Fig 5.12.K**).

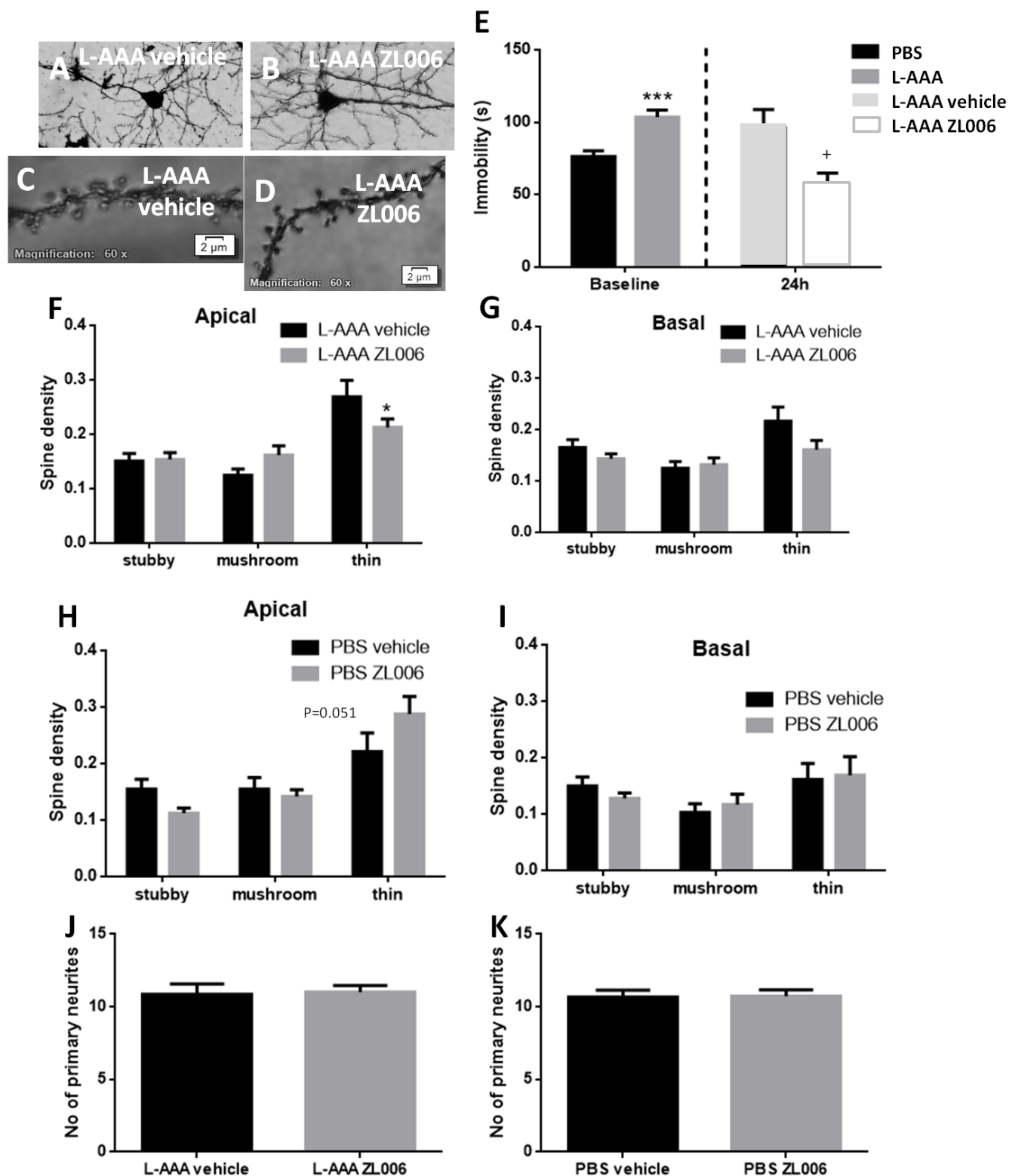


Figure 5.12: Effect of ZL006 in the FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

L-AAA (50µg/µl; 1µl) or PBS was administered to the PLC. 48 h later mice were exposed to the FST. The L-AAA group was subsequently subdivided into two groups and injected with either vehicle or ZL006 (10mg/kg). Mice were re-exposed to the FST 24 h later. Immediately following the second exposure to the FST, the animals were euthanised and brains were collected for golgi-cox staining. Representative picture for L-AAA vehicle

group (**A,C**), L-AAA ZL006 group (**B,D**). L-AAA increased immobility in the FST 48 h after administration (**E** left). In L-AAA-treated mice re-exposed to the FST 24 h after ZL006 reduced the immobility in the FST (**E** right). In the L-AAA-treated group, ZL006 didn't change spine density on apical dendrites but decreased thin spine density on basal dendrites (**F,G**). In the PBS treated group, ZL006 created a trend towards increased spine density on apical dendrites but didn't have any effect on basal dendrites (**H,I**). ZL006 didn't affect the number of primary neurites in L-AAA group (**J**) or in PBS group (**K**). (PBS vehicle or ZL006= 14 and 15 neurons; L-AAA vehicle or ZL006= 19 neurons, 3 mice). Data are expressed as mean $\pm$ SEM, (PBS vehicle= 10 mice, PBS ZL006= 10 mice, L-AAA vehicle= 9 mice, L-AAA ZL006= 9 mice for behaviour), *** $P < 0.001$ ** $P < 0.01$ and * $P < 0.05$ vs. Control PBS. ++ $P < 0.01$ vs L-AAA vehicle

7. Discussion

Results demonstrate that an IP injection of the PSD-95/nNOS inhibitor (ZL006) had no effect on dendritic spine density or the number of primary neurites. Swim stress (FST) reduced thin spine density on apical dendrites with a decrease in thin and mushroom spine density on basal dendrites and a decrease in the number of primary neurites. IP injection of ZL006 blocked FST-induced decrease in thin spine density on apical dendrites and thin and mushroom spine density on basal dendrites 24 h after administration. ZL006 was also able to block the depressive-like behaviour following re-exposure to the FST.

L-AAA decreased mushroom spine density on apical and basal dendrites 72 h after L-AAA administration with no effect on the number of primary neurites. L-AAA also produced depressive-like behaviours in the FST. Interestingly, FST combined with L-AAA drove the changes in the opposite direction and increased dendritic spine density. ZL006 was able to reduce thin spine density on apical dendrites and to exert antidepressant-related properties in the FST following L-AAA administration. Interestingly, those results differ from the literature as conventional antidepressant drugs increase stress-induced decrease in dendritic spine density in the PLC.

7.1. Effect of ZL006 on dendritic spine density and number of primary neurites in the PLC in the mouse

In this investigation, an IP injection of ZL006 in naive mice had no effect on spine density 24 h post administration. Brain plasticity refers to the brain's ability to adapt its structure and function depending on experiences (Kolb and Whishaw, 1998). Dendritic spines are related to changes in synaptic efficacy (LTP/LTD) and can be altered following new experiences or processes of learning and memory (Gipson and Olive, 2017). Therefore, these results suggest that ZL006 injected in mice in the absence of experiential learning doesn't influence the normal spine density level.

In addition, ZL006 had no effect on the number of primary neurites emerging from the soma 24 h post administration indicating that ZL006 is not capable of impacting the proximal network of dendrites emerging from the soma without an experiential stimulus.

Those results are in line with Doucet et al. (2013), who demonstrated that an injection of the inhibitor of the PSD-95/nNOS interaction IC87201 1 or 24 h prior to the TST did not produce any antidepressant-related activity suggesting that IC87201 without a first negative experience (exposure to the TST) doesn't display antidepressant-related properties. However, IC87201 or ZL006 when injected after a prior exposure to the TST or FST and re-exposed 24 h later to a second exposure displayed antidepressant-related activity suggesting that experience of the first exposure to the FST or TST is needed for IC87201 or ZL006 to produce an antidepressant response.

7.2. Effect of FST exposure on dendritic spine density and number of primary neurites in the PLC in mice

This study demonstrated that mice presented an increase in immobility on the second exposure of the FST. FST decreased thin spine density on apical dendrites and decreased thin and mushroom spine density on basal dendrites with a reduction of the number of primary neurites. The FST is sufficient to change spine dynamics and dendritic complexity. Depressive-like behaviour observed on exposure to the FST may be associated with the changes in dendritic spine density and dendritic complexity.

In animal models of depression, stress induces dendritic remodelling and synaptic spine reduction (Michelsen et al., 2007; Radley et al., 2008; Radley et al., 2013). Although the FST can be considered an acute stress, these results are in line with a loss of small spines (thin and stubby) in the PFC observed after CRS in rat (Bloss et al., 2011; Radley et al., 2008). In contrast, an IP injection resulted in increased thin and mushroom spine density on basal dendrites when compared to non-injected controls. This is in line with the literature showing that although stress mainly induces atrophy and spine retraction, it can have the opposite effect depending upon stress paradigm used (acute versus chronic or type of stressor) [for review, (Duman and Duman, 2015)]. In addition, in the PFC, social isolation increased the spine density of interneurons (Castillo-Gómez et al., 2017) and social defeat increased the maintenance of newly formed spines of pyramidal neurons 10 days post defeat (Shu and Xu, 2017) suggesting that a negative behavioural experience can also promote dendritic spine density.

7.3. Effect of ZL006 and TRIM on dendritic spine density and number of primary neurites in the PLC and associated changes in the mouse FST

As ZL006 did not impact dendritic spine density and neuronal complexity in the absence of an environmental stimulus, the effect of ZL006 on spine density was investigated in the FST stress paradigm. ZL006 and TRIM blocked the depressive-like behaviour observed following re-exposure to the FST. These results confirm those reported by Doucet et al. (2013) indicating that ZL006 and TRIM produce an antidepressant-related activity (Doucet et al., 2013) which may involve adaptive changes in neural plasticity following swim exposure.

In this experiment, in mice exposed to the FST, ZL006 blocked the reduction of thin spine density induced by the FST on apical dendrites of the PLC with no effect on the number of primary neurites. TRIM did not have any effect on FST-induced decrease in stubby spine density on basal dendrites but significantly blocked the effect of stress on the number of primary neurites. These results suggest that ZL006 is able to block the effect of a FST exposure on spine density. The ability of ZL006 to block the FST-induced reduction in thin spine density may be specifically explained by their dynamics as they are highly plastic and relatively transient spines (Bourne and Harris, 2007), are easily eliminated and responsible for acquisition of memory (Kasai et al., 2003, 2010) whereas stubby spines are more persistent and stable than thin spines (Sebastian et al., 2013).

As previously mentioned by (Licznarski and Duman, 2013; Duman and Duman, 2015), the mechanism of antidepressant drugs may involve spine and synapse remodelling. Therefore, ZL006 antidepressant properties may be linked to the attenuation of a stress-related decrease in spine density. Effects of ZL006 on stress-induced spine loss is also comparable with the effect of antidepressant drugs observed in the literature. A single dose of fluoxetine, ketamine or R-ketamine was able to attenuate the stress-induced spine loss in the PFC following social defeat in mice (Yang et al., 2015; Ma et al., 2016; Dong et al., 2017). Chronic fluoxetine administration also attenuated the stress-induced spine loss following chronic stress in the PFC in rat (Bessa et al., 2008).

The differential effect of ZL006 and TRIM on dendritic spine density and the number of primary neurites suggests that the dose of ZL006 (10mg/kg) and TRIM (50mg/kg), or their varied mechanisms of action, may influence spine density and neuronal complexity in different ways. It has been shown that thin spines only express a PSD once they start to enlarge, potentially becoming a mushroom spine, a phenomenon associated with LTP

(Yoshihara et al., 2009). As ZL006 inhibits PSD-95/nNOS interaction and rescued FST-induced reduction in thin spine, ZL006 is unlikely to work by directly influencing dendritic spines. The results suggest that the mechanism of action of ZL006 may require an additional action other than inhibiting the interaction between PSD-95/nNOS. Recent investigations indicate that the mechanism of action of ZL006 may not involve direct binding to the extended nNOS-PDZ domain as originally proposed but instead could bind to other parts of the large and complex 321-kDa homodimer nNOS protein or to other proteins affecting the nNOS/PSD-95 interaction (Bach et al., 2015).

7.4. L-AAA delivery to the PLC reduces dendritic spine density and the number of primary neurites in the PLC in mice

72 h after L-AAA-induced astrocytic toxicity, L-AAA had no effect on the number of primary neurites but decreased mushroom spine density on apical and basal dendrites compared to PBS controls. Those results suggest that dysfunctional astrocytes following L-AAA can affect mature spines in the PLC in a similar way as previously reported following CUS (Michelsen et al., 2007; Li et al., 2011; Radley et al., 2013).

The effect of FST-induced stress differs by affecting thin spines whereas 72 h after L-AAA administration thin spines were not affected. This difference may be due to the second timing of exposure to the FST as brains were collected 1h after. The specificity of action of L-AAA on mushroom spines 72 h after L-AAA administration *in vivo* and thin and mushroom spines in hippocampal transfected neurons following the application of astrocyte L-AAA CM treatment for 24 h *in vitro* (from chapter 3) is most likely related to time. As thin spines are more flexible, they may recover earlier from L-AAA administration compared to mushroom spines. Thin spines early reduced may also manifest on a decrease of mushroom spines formation after 24 or 48 h.

To our knowledge, it is the first time that L-AAA has been shown to reduce dendritic spine density. Lima et al. (2014) demonstrated that a high dose of L-AAA (20µg/µl, 5µl, 1 injection) injected in the PFC had no effect on dendritic spine density in the rat PFC. However, they showed that L-AAA had an effect on neuronal complexity (dendritic length). Because of time and technical constraints, our analysis was only focused on the number of primary neurites. Therefore it is not possible to directly compare the effect of L-AAA on neuronal complexity to Lima et al. (2014). However, in the future, it would be of interest to perform a complete Sholl analysis.

7.5. Effect of ZL006 in the mouse FST and associated changes in dendritic spine density and number of primary neurites in the PLC following L-AAA-induced astrocytic toxicity

This research previously demonstrated that the FST or L-AAA administration decreased spine density. However, FST combined with L-AAA drove changes in spine density in the opposite direction and increased stubby, mushroom and thin spine densities on apical dendrites and stubby and mushroom spine densities on basal dendrites compared to their non FST counterparts. These results indicate that a stress experience (FST) counteracts the decrease in spine density induced by L-AAA administration. These results might explain why Lima et al. (2014) didn't find any effect of L-AAA on spine density after L-AAA administration (20µg/ul; 5µl; 1 injection) in the PFC of rats. L-AAA was combined with either the ASST or the reversal memory task in the water-maze that may have interfered with the effect of L-AAA in a similar way to the impact of the FST in the present investigation.

This study also reproduced the depressive-like behaviour in the FST observed 48 h after L-AAA administration in the PLC (50µg/ul; 1µl; 1 injection) reported in chapter 4. This is in accordance with the depressive properties attributed to L-AAA reported in rats (100µg/ul; 0.5µl; 2 days) and in mice (100µg/ul; 0.6µl; 2 days) (Banasr and Duman, 2008; Lee et al., 2013). Taking into consideration that ZL006 requires a prior exposure to the FST to observe antidepressant-related properties (Doucet et al., 2013), mice were injected with either ZL006 or vehicle after a first exposure to the FST and were re-exposed 24 h later to test the effect of ZL006 following L-AAA-induced astrocytic toxicity in mice. There was no increase in immobility upon second exposure in the L-AAA group compared to immobility scored on initial exposure, suggesting a plateau of immobility. Moreover, ZL006 reduced immobility on the second exposure to the FST in the L-AAA-treated group 24 h after treatment. These results are in agreement with the antidepressant-related properties of ZL006 in naive mice (Doucet et al., 2013). To our knowledge, this is the first time that ZL006 was shown to decrease immobility in the L-AAA-induced astrocytic toxicity model.

ZL006 has been previously shown to block FST and L-AAA induced- reductions in thin spine density. However ZL006 when combined with L-AAA administration and the FST was found to decrease thin spine density on apical dendrites compared to animals injected with vehicle, suggesting that ZL006 regulates the increase in spine density induced by L-AAA/FST. Moreover, ZL006 did not have any effect on the number of primary neurites.

In the FST combined with PBS group, ZL006 had no effect on dendritic spine density although there was a trend towards increase of thin spine density on apical dendrites. ZL006 didn't affect the number of primary neurites and did not show antidepressant-related properties in the FST. These results could be related to the effect of the surgery described in chapter 4 predisposing to astrocytic reactivity in the area of injection.

Reduced GFAP immunoreactivity observed following L-AAA administration in the PLC are in line with post-mortem studies in MDD patients showing that there are significant reductions in glial density in the fronto-limbic areas (Rajkowska and Miguel-Hidalgo, 2007) and higher glutamate levels (Küçükbrahimoğlu et al., 2009). Results from this investigation suggest that ZL006 is able to attenuate L-AAA-induced depressive-like behaviour associated with a decrease of spine dynamics. Antidepressant drugs have been shown to regulate synapse remodelling (Licznarski and Duman, 2013; Duman and Duman, 2015). Here this research revealed a dual role of ZL006 in spine dynamics. ZL006 acts as a modulator by decreasing or increasing spine density to regulate stress related changes in dendritic spines either in the presence or absence of astrocytic dysfunction.

Interestingly, stress (footshock) leads to an increase of glucocorticoid release and has been shown to increase depolarization-evoked glutamate release in the PFC (Musazzi et al., 2010). Taking into consideration the negative effect of excessive glutamatergic transmission on neurons, the neuronal atrophy and spine loss observed in this investigation might be mediated by glutamate release following FST. Spine retraction has been linked to glutamate. The retraction of small spines is NMDA-R-dependent while that of large spines requires both NMDA-R and metabotropic glutamate receptors and have a higher sensitivity to glutamate (Oh et al., 2013). Taking into consideration that L-AAA impairs the glutamate metabolic cycle; this may explain the long lasting effect of L-AAA on mushroom spines.

In addition, chronic antidepressant treatments for 2 weeks prior the footshock stress were able to prevent the excessive glutamate release in rat (Musazzi et al., 2010). Therefore the protective effect of ZL006 and TRIM from stress and from L-AAA-evoked changes in dendritic spines might be linked to their regulation of glutamate levels observed following FST exposure.

8. Conclusion

Our study confirms that ZL006 has antidepressant-like properties following L-AAA-induced astrocyte toxicity in mice. ZL006 appears to act as a regulator of spine density level and was able to attenuate FST-induced decrease in dendritic spine density in the PLC. ZL006 was also able to attenuate the increase in dendritic spine density following FST combined with L-AAA administration in the PLC. The results suggest that the antidepressant-related properties of ZL006 may be linked to a regulation of spines in brain circuits affected by exposure to the FST. To have a better understanding of the action of L-AAA, it would be necessary to measure glutamate levels and assess the effect of ZL006 following L-AAA administration without exposure to the FST. ZL006 should also be tested earlier after L-AAA administration to determine if co-treatment of L-AAA and ZL006 can impact on immature thin spines. It would also be of interest to test ZL006 in alternative stress models to confirm these findings.

Chapter 6

General discussion and future directions

1. Cellular/animal models of astroglia dysfunction

There is a need to develop cellular and animal models of astroglial impairments and dysfunction. Recent interest has emerged in evaluating the role of astrocytes in a range of neurodegenerative and psychiatric disorders (Cotter et al., 2001; Anderson et al., 2003; Panickar and Norenberg, 2005; Maragakis and Rothstein, 2006; Rajkowska and Miguel-Hidalgo, 2007; Takano et al., 2009; Verkhratsky et al., 2010; Halliday et al., 2011). A number of post-mortem studies in MDD patients have shown that there are significant reductions in glial density in the fronto-limbic areas (Rajkowska and Miguel-Hidalgo, 2007), the subgenual cingulate cortex (Williams et al., 2012), the amygdala (Bowley et al., 2002) and the left hippocampus (Cobb et al., 2016). The decrease of astrocytes observed in MDD has been associated with alterations in central glutamate as reported with MRS in the PFC (Yildiz-Yesiloglu and Ankerst, 2006; Luykx et al., 2012; Miladinovic et al., 2015). Glutamate under normal physiological conditions promotes neurite outgrowth and synaptogenesis. However, the functional implications of the astrocytic impairments on glutamate transmission are not well understood. Taking into account the role of astrocytes in the trophic support of neurons and that higher glutamate levels may exert excitotoxic effects on neurons or contribute to neuronal atrophy, further investigation is required.

L-AAA has received particular attention in recent years as an effective method of selectively inducing impaired astrocytic function (Olney et al., 1980; McBean, 1994; Brown and Kretzschmar, 1998). Hypothetically, L-AAA reduces ATP production and impairs glutamate transport in astrocytes with a possible increase of glutamate transmission through the NMDA-R, which contributes to neuronal atrophy, synapse and spine loss. This is in line with clinical studies, in MDD patients where depression is associated with a reduction in volume of a number of brain regions (Rajkowska et al., 1999; Bianchi et al., 2005; Warner-Schmidt and Duman, 2006). Using electron microscopic stereological analysis, the number of spine synapses was decreased in the PFC (Kang et al., 2012) and the expression of synapse-related genes was altered in the hippocampus (Duric et al., 2013). Reduced dendrite and spine densities were observed in the CA3 region of the hippocampus using light microscopy (Soetanto et al., 2010) and reduced dendritic spine density and diminished arborisation of apical dendrites were reported in the subiculum in a small group of mixed subjects with bipolar disorder or depression (Rosoklija et al., 2000).

This investigation aimed to refine a mouse model of astrocytic dysfunction by injection of a single dose of L-AAA using a microcapillary in the PLC to limit astrocytic activation associated with the injection. The results showed that a single injection of L-AAA into the PLC (50µg) in mice is sufficient to induce a decrease in astrocytic markers (GFAP and S100b). L-AAA also induces depressive-like behaviours without affecting the immunoreactivity of the neuronal markers (NeuN and DCX) or the microglial marker (Iba1). Moreover, L-AAA reduced mature spine density on apical and basal dendrites of neurons. However, in the *in vitro* studies, CM-L-AAA reduced thin and stubby spines after 24 h but in the *in vivo* model, L-AAA only reduced mushroom spines after 72 h. This difference might be linked to the time difference as mushroom spines are more mature compared to thin and stubby spines. In addition, *in vitro*, CM L-AAA decreased the number of primary neurites after 24 h whereas it didn't have any effect *in vivo* after 72 h. The difference observed might be due to the time difference or to the complexity on the *in vivo* system as neurons are parts of a system. The animal model used in this research allowed to measure behavioural and post-mortem changes, helping to bridge the gap with clinical studies in MDD patients which are mostly focused on connectivity and with limited assessment post-mortem (**Fig 6.1**).

In addition, it has been shown in the literature that L-AAA affects learning and memory. In rats, a single high dose of L-AAA into the mPFC (20µg/µl, 5µl, 1 injection) affected performance in the attentional set-shifting test (AASST) and the working memory in the water-maze. These results are of importance given the role of astrocytes in the tripartite synapse and therefore memory and in the context of using agents influencing the NMDA receptor signalling in this thesis.

2. New antidepressants targeting the glutamatergic pathway

There is a need for the development of new antidepressants. Despite the advances that have been made, there are still unmet clinical needs to be addressed (e.g, treatment resistance to conventional drugs, slow onset of action of existing drugs and side effects such as sexual dysfunction and weight gain) (Schechter et al., 2005). Between 50 and 60% of patients are resistant to current anti-depressant treatments (Trivedi et al., 2006).

The NMDA-R antagonist ketamine has demonstrated rapid antidepressant effects in resistant patients. Ketamine is currently limited due to its adverse effects. Ketamine has sedative and psychotomimetic side effects, and repeated exposure increases the risk of severe psychosis, dissociative episodes and euphoria (Machado-Vieira et al., 2009). Therefore, there is a need

to develop more selective drugs. Although the effect of chronic administration of PSD-95/nNOS inhibitor hasn't been examined yet, as they target selectively the NMDA-R/NO signalling pathway compared to ketamine which acts as an antagonist of the NMDA-R, we do not expect to observe the same side effects. Moreover, behavioural measures associated with NMDA-R antagonism and nNOS inhibitors have significant side effects, such as spatial and episodic (source) memory, aggressive behaviour, reduced locomotion, and decreased motor coordination (Dzoljic et al., 1997; Kemp and McKernan, 2002; Volke et al., 2003; Zhou et al., 2010; Doucet et al., 2013; Smith et al., 2016), not present with PSD-95/nNOS interaction inhibitors (Doucet et al., 2013; Lee et al., 2015; Smith et al., 2016). These results indicate that ZL006 disrupts PSD-95/nNOS without causing adverse effects and support its potential for drug development (Doucet et al., 2012).

Taken together, those previous results indicate that ZL006 might be advantageous over current antidepressant strategies and ketamine. However, it would be interesting in the L-AAA-induced astrocytic dysfunction model to compare the effect of ZL006 to current antidepressant drugs as so far as we know, we are the first one to assess the effect of a potential antidepressant (ZL006) in this model. Doucet et al. (2013) reported that IC87201 and ZL006 produce antidepressant-like responses in the FST and TST following a single administration in mice. By contrast to the tricyclic antidepressant imipramine, the effects are not observed 1 h following drug administration but are apparent 24 and 72 h later. Furthermore prior exposure to the TST or FST is required in order to observe the antidepressant-related activity. Similar delayed and sustained antidepressant-like effects were observed following TRIM and ketamine in the TST. In addition, as conventional antidepressants are chronically administered, it would be interesting to assess the effect of chronic ZL006 administration and compare its effect to conventional antidepressants and ketamine in a chronic stress model or L-AAA-induced astrocytic dysfunction model. However, the use of the L-AAA-induced dysfunction model might be limited due to the recovery of both depressive-like behaviours and GFAP immunoreactivity decrease after a week. Another study from our lab showed that both TRIM and ZL006 decreased immobility time in Wistar Kyoto rats in the FST (Sherwin et al., 2017b) although one other study didn't show any effect of a single or chronic administration of ZL006 on depressive-like behaviours in Flinders Sensitive Line rats, Flinders Resistant Line rats, Wistar Kyoto rats, Wistar Hanover rats, Sprague Dawley rats, B6NTac mice (Tillman et al. 2017).

As the neuronal complexity and structural plasticity of neurons is potentially impaired in depression and the brain is plastic with capacity to repair itself, restoring neural integrity and connectivity may represent a novel therapeutic approach (Merzenich et al., 2014; Opler et al., 2014). The glutamatergic NMDA-R activates the PSD-95/nNOS pathway leading to the activation of NO which plays a pivotal role in synaptic transmission and plasticity (Volianskis et al., 2015). Uncoupling nNOS from the NMDA-R with PSD-95/nNOS inhibitors IC87201 and ZL006 rescues NMDA-glycine-induced neuronal atrophy (Doucet et al. 2015). However, there is an alternative pathway: NOS1AP coupled with nNOS mediates the p38 MAPK cell death pathway (Li et al., 2013), although further work is necessary. Furthermore, targeting the NMDA-R/NO signalling pathway attenuates reductions in neuronal complexity associated with impaired astrocytes (chapter 3).

Results from this investigation demonstrated that targeting the NMDA-R, nNOS or PSD-95/nNOS interaction partially protects against neuronal atrophy or synapse loss induced by CM obtained from L-AAA-treated astrocytes providing insights into the potential of NMDA-R antagonists and downstream inhibitors of the PSD-95/nNOS interface as a strategy for attenuating neuronal outgrowth impairments related to dysfunctional astrocytes. Therefore, discretely targeting the NMDA-R/NO pathway to block alterations in the structural plasticity of neurons related to stress or astrocytic impairment may be considered a potential approach to promote antidepressant action.

3. Inhibitors of NMDA-R/NO signalling regulate dendritic spine density

The brain is malleable and constantly adapts to the changes in its environment via pre-synaptic neurotransmitter release, post-synaptic receptor number, as well as the nature, strength, or number of spines and synaptic contacts (Bliss and Lømo, 1973; Citri and Malenka, 2008). Resilience and plasticity following stress is a key aspect of a healthy brain. However, in pathological conditions, there is a loss of resilience which may be associated with deficits in neuronal complexity and dendritic spines [for review, (McEwen, 2016)]. There is emerging evidence linking stress-related reductions in dendritic spine density homeostasis, dendrite arborisation and neuronal complexity with the pathophysiology of stress-related psychiatric disorders such as depression (Sato, 2013; Duman and Duman, 2015). In addition, astrocytic dysfunction exerts similar negative effects on neuronal complexity as those observed following stress.

Unlike neuronal loss, which cannot be reversed, atrophy and synaptic loss can be rescued by the enhancement of neurite outgrowth and synaptogenesis. Higher outgrowth increases the neuronal network and synaptic space as dendritic branches provide the physical substrate where synapses are formed. It can facilitate the development of new synaptic connections as well as the re-stabilisation of vulnerable synapses. Therefore, alterations in the structural plasticity (complexity and dendritic spines) of neurons have been proposed as a putative mechanism underlying the therapeutic properties of antidepressants.

On their own, swim stress (FST) and L-AAA administration decreased dendritic spine density in the PLC. This effect was blocked by ZL006 treatment. Conversely, the combination of L-AAA and FST increased dendritic spine density in the PFC. This effect too was opposed by ZL006 treatment. It appears therefore that ZL006 opposed induced changes in dendritic spines in either direction and serves to stabilise spines in the face of environmentally imposed conditions (**Fig 6.2**). *In vitro*, results showed that ZL006 is able to rescue L-AAA CM-induced loss of synaptic puncta. Taking into account that dendritic spine densities are involved in synapse formation, *in vitro* and *in vivo* results appear to both show a rescue from ZL006 following L-AAA-induced changes.

In addition, although ZL006 was able to rescue L-AAA CM-induced decrease in the number of primary neurites, it had no effect on number of primary dendrites *in vivo*. It is important to mention that L-AAA did not have any effect on the number of primary neurites *in vivo* but the differences might be associated to the time difference and the complexity of the neuronal network in the brain compared to neuronal cell culture *in vitro*.

Taken together, those results suggest that ZL006 is able to create depressive-like behaviour and to stabilise changes in neuronal morphology/spine density following stress or dysfunctional astrocytes suggesting favorable therapeutic outcome of PSD-95/nNOS disruption in the treatment of depression and resistant patients.

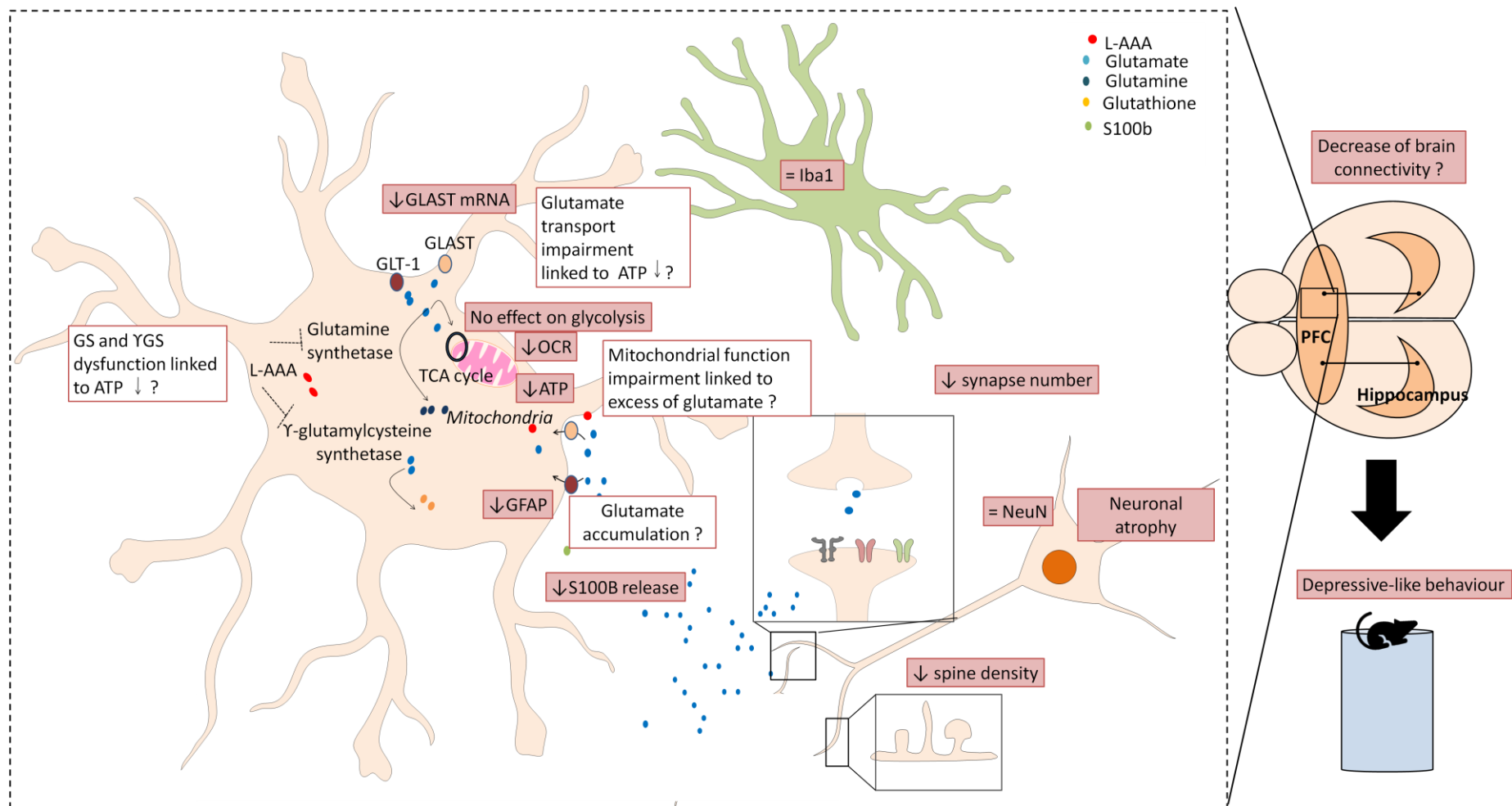


Figure 6.1: L-AAA-induced astrocytic toxicity and potential mechanisms mediating changes in neuronal structure and plasticity

L-AAA accumulates inside astrocytes via the glutamate transporters GLAST and GLT-1. L-AAA accumulation impairs glutamate transport, leading to an extracellular accumulation of glutamate which promotes neuronal atrophy, synapse and spine loss. L-AAA inhibits GS, leading to an accumulation of glutamate affecting mitochondrial respiration and ATP-linked mitochondrial respiration. L-AAA also inhibits γ -glutamylcysteine synthetase, leading to a decrease in glutathione production. L-AAA also reduces the astrocytic markers (GFAP and S100b) but doesn't affect the neuronal marker (NeuN) or microglial marker (Iba1). Changes observed in spine density may be associated with network connectivity impairments related to changes in behaviour (e.g. connection between the PFC and the hippocampus). Reduced neuronal complexity, synapse formation and spine density and depressive-like behaviours following L-AAA are partially reversed after treatment with inhibitors of the NMDA-R/NO signalling pathway.

4. Limitations and future directions

Several studies could be carried out to further elaborate upon the results presented in this thesis.

Variability between different replicates in cell culture can be problematic as neurite outgrowth is sensitive to small differences in culture conditions between experiments (Blackmore et al., 2010). In addition, image acquisition can be time-consuming and small changes in outgrowth or retraction may not be visualised by fluorescent microscopy. The use of β III-tubulin as a neuritic marker to visualise neurons does not distinguish between axons and dendrites. To quantify the neuronal complexity of individual neurons *in vitro*, cortical immature neurons (DIV3) were used for the sholl analysis. Although Doucet et al. (2015) demonstrated that immature neurons at DIV3 express PSD-95 and nNOS, more mature neurons have higher sensitivity to glutamate than immature neurons (Monnerie et al., 2003). Therefore, it would complement the current study to determine neuronal complexity in mature neurons. For this purpose, the complexity of mature neurons can be visualized with transfection of plasmid incorporating GFP DNA in neurons as a low percentage of the total neurons express GFP and thus individual neurons can be visualised and assessed. This thesis described the use of hippocampal transfected neurons to quantify dendritic spine density because, as pilot experiments, GFP transfection attempts in cortical neurons were unsuccessful.

Sholl analysis is a widely used method for the measurement of neuronal complexity. In this thesis, we used a semi-automatic analysis adapted from the classic sholl analysis (Gutierrez & Davies, 2007). It requires analysis of isolated individual neurons. This does not accurately reflect the physiological conditions where neurons are arranged in neuronal networks *in vivo*. Therefore, neurite outgrowth in a neuronal network should be investigated. In this regard, live cell Ca^{2+} imaging has recently been used to examine neuronal network activity *in vitro* (Verstraelen et al., 2014) using this technique to quantify the effect of L-AAA would be of interest to better mirror to *in vivo* conditions.

This thesis demonstrated that L-AAA induces astrocytic dysfunction with reduced mitochondrial respiration and ATP production which is necessary for glutamate transport. This decrease of mitochondrial respiration may be due to glutamate accumulation inside the astrocytes as previously mentioned (Yan et al., 2017). Therefore, a real measure of glutamate levels is needed to confirm the link between any potential L-AAA-induced astrocytic

impairment in glutamate transport and the neuronal atrophy or dendritic spine loss observed. It would be of interest to measure glutamate uptake in astrocytes treated with L-AAA. Uptake of ^3H aspartate is commonly used instead of glutamate as it is more stable *in vitro* and *in vivo* on fresh brain tissue as previously mentioned (Dumont et al., 2014). It is also possible to measure glutamate levels using MRS or glutamate sensors implanted in the brain composed of glutamate-binding proteins coupled with a fluorescence readout with a signal assigned to glutamate-evoked as previously described (Marvin et al., 2013). Quantifying glutamate following L-AAA treatment would provide for a better understanding of its mechanism of action.

In addition, because of time constraint and limitations of software, this investigation analysed the number of primary neurites only, as a measure of neuronal complexity *in vivo*. However, it would be of interest to measure the dendritic length and the numbers of secondary branches to more fully quantify neuronal complexity. For this purpose, improved software, e.g. Neurolucida instead of Neuron studio may be applied to quantify those parameters on golgi-cox stained slices. Another option would be to transfect post-mortem slices with plasmid incorporating GFP DNA as described in chapter 3 *in vitro*. Lastly, delivery of a CAG-GFP retro-virus directly in the brain would allow for measures of neuronal complexity and spine density to be obtained in individual neurons as previously described (Zhao et al., 2006). In addition to assessing individual neuronal complexity, as neurons communicate between each other, it would be of interest to assess neuronal connectivity with resting state fMRI or EEG such as connectivity between the PFC and the hippocampus following L-AAA delivery to the PLC.

The number of astrocytes was quantified by GFAP positive cell number using a method adapted from Gosselin et al. (2009). However, GFAP staining is a limited technique, as in normal conditions the majority of astrocytes in grey matter will express relatively small amounts of GFAP protein. GFAP immunoreactivity increases when astrocytes are activated in response to acute trauma. Only a small subpopulation of astrocytes has been reported to proliferate in response to acute trauma in the cerebral cortex (Bardehle et al., 2013). Therefore, changes in GFAP positive cell number reported in this thesis are not fully representative of changes in astrocyte cell number but instead better reflect astrocytic function and morphology. It would also be of interest to assess astrocytic complexity as astrocytes may retract following L-AAA treatment decreasing the number of receptors and impairing BBB permeability.

In addition, there is a decrease in S100b released from astrocytes observed after L-AAA administration in the PLC. Taking into account that S100b is increased in the plasma of depressed patients, quantifying S100b in the plasma of animals treated with L-AAA may help to further illustrate the effect of L-AAA in the brain and measure BBB permeability.

Although we didn't find any difference in the number of microglia cells, it would be interesting to look at microglia morphology following L-AAA treatment as those cells seem more reactive compared to PBS treated group.

Another limitation of this project relates to the temporal nature of L-AAA-induced effects. Changes in behaviour and astrocytic markers were transient (72 h after L-AAA administration) with a recovery evident 7 days after the injection. Therefore, behaviour has to be performed a short time after the surgery/injection and different animals were used for each test to avoid potential interferences between tests.

In this investigation, we assessed the effect of inhibitors of the NMDA-R/NO signalling pathway *in vitro* but only focused on ZL006 and TRIM *in vivo*. Testing the effect of other drugs or peptides targeting PSD-95/nNOS interactions (Doucet et al. 2012) in FST-induced or L-AAA-induced spine density impairment would be of interest to confirm the effect of ZL006 and the impact of targeting the NMDA-R/NO signalling pathway. In addition, in this research, we only focused on the PLC. To confirm the effect of ZL006, it would be of interest to assess its effect on neuronal complexity in another brain region involved in stress and mood regulation e.g. the hippocampus/amygdala, but also as a control to assess its effect in brain regions not involved in mood regulation/stress e.g. the striatum or caudate nucleus.

Determining a time-course effect of L-AAA on spines would also be of interest. Here this research demonstrated that L-AAA affects mushroom spines 72 h after administration *in vivo* although a pilot study *in vitro* on primary hippocampal neurons suggests that it affects thin and mushroom spines 24 h after the application of CM from L-AAA-exposed astrocytes. It would be of interest to see if after a shorter time after L-AAA administration (24 h), ZL006 can reverse L-AAA-induced spine density impairment.

Lastly, this study demonstrated that CM from healthy or dysfunctional astrocytes affects neuronal outgrowth and synapse number. Determining by high content analysis which molecules are released by astrocytes into the CM that impact neurons in a physiological or disease-salient context would help to further understand astroglia-neuronal interactions (Buchser et al., 2004).

Appendix

SLICK-H mice

1. SLICK-H transgenic mice

1.1. Breeding strategy

To produce SLICK-H tg mice, an in vitro fertilization (IVF) was performed (**Fig 7.1**). The sperm of the Thy1-Cre-YFP males was first frozen to bank away the line for sperm cryopreservation. IVF was subsequently performed with oocytes obtained from C57BL6/J females. The resultant embryos were retrieved and implanted into pathogen-free mice. The offspring was genotyped to determine the genetic background. We used a harem-style breeding strategy for males: one male Tg(Thy1-cre/ERT2,-EYFP)HGfng was housed with several females whereas females Tg(Thy1-cre/ERT2,-EYFP)HGfng were only housed with one male at the time. The resulting Tg(Thy1-cre/ERT2,-EYFP)HGfng mice were hemizygous for Tg(Thy1-cre/ERT2,-EYFP)HGfng. Hemizygous is different from heterozygous as it is characterised by having one or more genes without allelic counterparts. The resulting embryos were 50% hemizygous and 50% wild type for the genes.

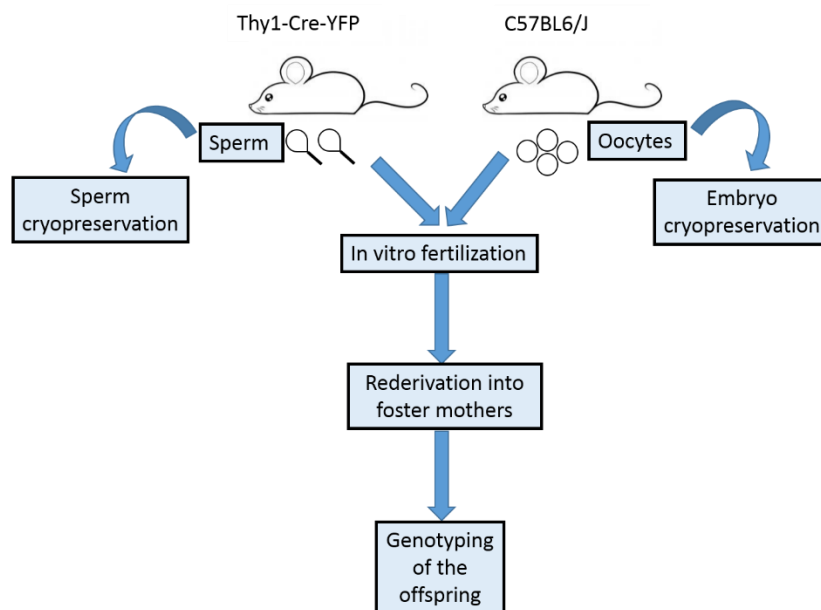


Figure 7.1: IVF protocol

To produce Tg(Thy1-cre/ERT2,-EYFP)HGfng mice, an in vitro fertilization (IVF) was performed. The resultant embryos were subsequently retrieved and implanted into pathogen free mice.

1.2. Cre/LoxP system

The transgene was designed with a creERT2 fusion gene, two copies of the mouse Thy1 promoter, and an enhanced YFP (EYFP) cDNA sequence. The creERT2 fusion gene (cre/ERT2) is Cre recombinase fused to a G400V/M543A/L544A triple mutation of the human estrogen receptor ligand binding domain. Each Thy1 promoter region is a modified

regulatory region of the murine thy1.2 gene (extending from the promoter to the intron following exon 4, excluding exon 3 and its flanking introns). The two copies are placed "back to back" to function bidirectionally; one copy drives expression of CreERT2, the other copy drives expression of EYFP. When tamoxifen is administered at a desired time point in embryonic development or adult life, the Cre-recombinase enables an excision of the two Lox-P sites and an expression of the gene of interest (fluorescent protein). The Cre reporter strain contains loxP sites in combination with visible (fluorescent) marker proteins used to trace Cre recombination success (**Fig 7.2**). The Cre-ERT2 fusion protein consists of Cre recombinase fused to a triple mutant form of the human estrogen receptor which does not bind its natural ligand (17 β -estradiol) at physiological concentrations but will bind the synthetic estrogen receptor ligands 4-hydroxytamoxifen (tamoxifen). Restricted to the cytoplasm, Cre-ERT2 can only gain access to the nuclear compartment after exposure to tamoxifen.

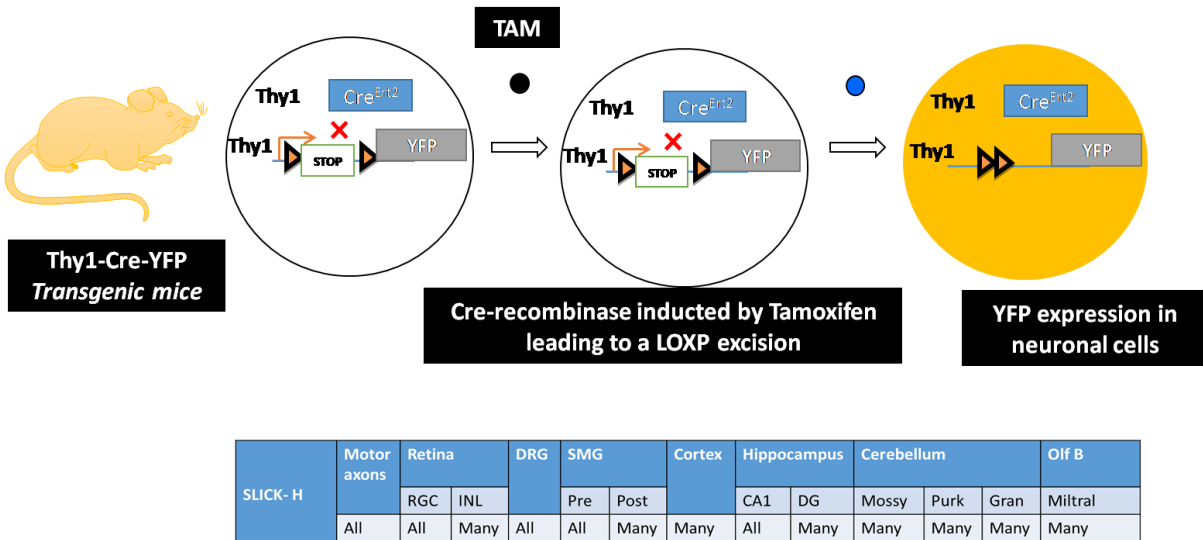


Figure 7.2: Thy1-Cre-YFP mouse

The Thy1-Cre-YFP mouse strain from the Jackson laboratory is genetically engineered for the induction of YFP under the Thy1 neuronal promoter to render YFP expression specifically in neuronal cells. This construct is based on the inducible Cre system which contains a transgene that expresses a modified form of Cre-recombinase that is non-functional until an inducing agent, tamoxifen, is administered to the animals permitting recombination of YFP expression in neuronal population. YFP is expressed in projection neurons. Projection neurons are glutamatergic neurons characterized by a typical pyramidal morphology that transmit information between different regions of the neocortex and to other regions of the brain. Few denotes < 10% labeled; many denotes 10 – 90 % labeled and all denotes > 90% labelled RGC = retinal ganglion cell, INL = inner nuclear layer, DRG = dorsal root ganglion, SMG = submandibular ganglion, pre =presynaptic post = post synaptic, DG = Dentate granule cells, Mossy = cerebellar mossy fibers, Purk = cerebellar purkinje cells, Gran = cerebellar granule cells Olf B = Olfactory Bulb, Mitral = mitral cells [table of YFP expression adapted from Young et al., (2008)].

2. Methods

2.1. Drug preparations

Tamoxifen was injected intraperitoneally to SLICK-H mice for 5 days in an injection volume of 10ml/kg. Tamoxifen was prepared weekly and stored at -20°C.

Table 7.1 Tamoxifen preparation for IP injection

Compound name	Molecular weight (g/mol)	Injection volume	Concentration	Dissolved in:
Tamoxifen	372	10ml/kg	150mg/kg	Corn oil

2.2. Stereotactic delivery of L-AAA in the PLC

Male/female 12 week old SLICK-H mice received L-AAA (50µg/µl; 1µl; 0.2µl/min) or PBS administration into the PLC. 72 h after L-AAA administration, mice were transcardially perfused with heparinised PBS (0.1M, pH 7.4; heparin 1ml at 5000UI/ml) for 3 min, and then with PFA (4% PFA in PBS) for 3 min 30 sec with the abdomen clamped. Following transcardial perfusion, brains were sliced into serial frontal sections (150µm thick) with a vibratome.

2.3. YFP expression quantification in SLICK-H mice

Following transcardial perfusion, brains were sliced into serial frontal sections (150µm thick) with a vibratome. The sections were washed 3 times for 10 min in PBS and mounted onto gelatine-coated slides and any excess liquid was removed. Vectashield mounting medium DAPI (Vector laboratories) was applied and coverslips were placed on the slides and sealed with nail varnish. The sections were stored at 2-4°C until imaged with the confocal microscope. This investigation aimed to determine whether it was possible to assess neuronal complexity and spine density on those mice with a range of different techniques: confocal or multi-photons microscope, use of the deconvolution software Imaris to reduce the noise in the picture, use of antibody directed against GFP to improve the signal (1:2000, Thermofisher, Life Technologies, Carlsbad, CA, USA). Detection of GFP was enabled with Alexa dye conjugated antibodies (1:500, Molecular Probes, Eugene, OR, USA, Thermofisher, Waltham, MA, USA) and nuclei were visualized with Hoechst-33342 (1:1000) and finally with the CLARITY technique. Clarity technique removes lipids from the tissue. This process creates a tissue-hydrogel hybrid that is physically stable, that preserves fine structure, proteins and nucleic acids, and that is permeable to both visible-spectrum photons and exogenous macromolecules (Chung and Deisseroth, 2013).

In addition, image stacks of the regions of interest were acquired with a 20x objective using a Leica confocal SP8 microscope with a zoom of 0.7. The total number of YFP positive cells was counted in the PLC in 3 squares of 250µm by 250µm per region in the right and left hemispheres of the brain. The number of cells was averaged and expressed as the number of cells per mm².

3. Results

3.1. Assessing neuronal complexity and spine density with SLICK-H mice

The aim of this study was to assess whether it is possible to assess neuronal complexity and spine density in the PLC of SLICK-H mice previously described by Young et al., (2008). 72 h after L-AAA (50µg/µl; 1µl; 0.2µl/min) or PBS administration, YFP positive neurons were visualized using a confocal microscope (**Fig 7.3.A,B**). However, it wasn't possible to visualise spine density or complete dendritic architecture to quantify neuronal complexity in the PLC. Although the quality of the picture was better when the noise was decreased by deconvolution treatment with Imaris software, determining spine density/neuronal complexity was still impossible (**Fig 7.3.C**).

To obtain a better quality picture, YFP positive neurons were visualised using a multi-photon microscope (**Fig 7.3.D**). The neuronal cell bodies and the main axon were clearly visualised but differentiation of the individual neuronal branches wasn't possible. The next step was to improve the signal by using an antibody directed against GFP which also binds YFP (**Fig 7.3.E,F**). Although there was a signal improvement, it wasn't enough to determine spine density or neuronal branching. Lastly, CLARITY was used on a SLICK-H section and was visualised under a confocal microscope (**Fig 7.3.G,H**). Although neuronal cell bodies were easy to visualise, the differentiation of the dendrites and visualisation of spines was not possible because of background and poor signal.

To conclude, visualizing spine density and neuronal complexity in the SLICK-H mice wasn't possible. As the neuronal cell bodies were easy to visualise, SLICK-H mice were used to quantify viable neurons after L-AAA treatment. The initial plan was to perform the spine and neuronal complexity analysis on SLICK-H mice. However, as this was deemed unfeasible technically, the golgi-cox staining impregnation technique was adopted in subsequent experiments.

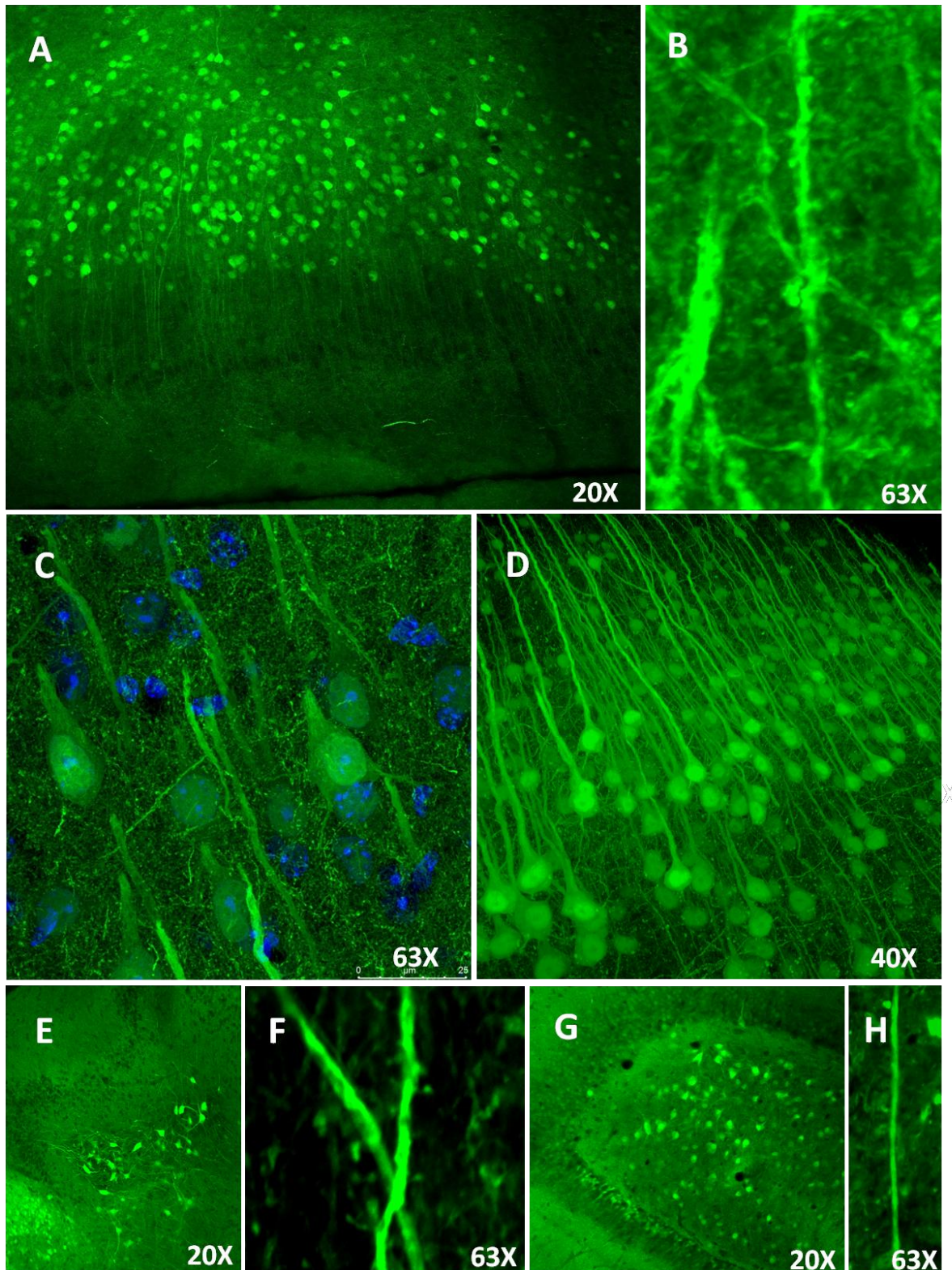


Figure 7.3: YFP positive neurons from SLICK-H mice

YFP positive neurons (complexity and dendritic segments) (Blue: DAPI; Green: YFP) were visualized using the confocal microscope (**A,B**), after deconvolution treatment with Imaris software (**C**), using the multi-photon microscope (**D**), with antibody directed against GFP in the PLC (**E,F**) or using CLARITY (to remove lipids by using acrylamide-based hydrogels) with the confocal microscope in the hippocampus (**G,H**).

3.2. Effect of L-AAA on viability of neurons in SLICK-H mice

Student's T-test showed no effect of L-AAA administration (50µg/µl; 1µl) on YFP positive neurons in the PLC 72 h after L-AAA administration, [$T_{(13)}=0.17$, $P=0.86$] when compared to vehicle-treated controls (Fig 7.4.C).

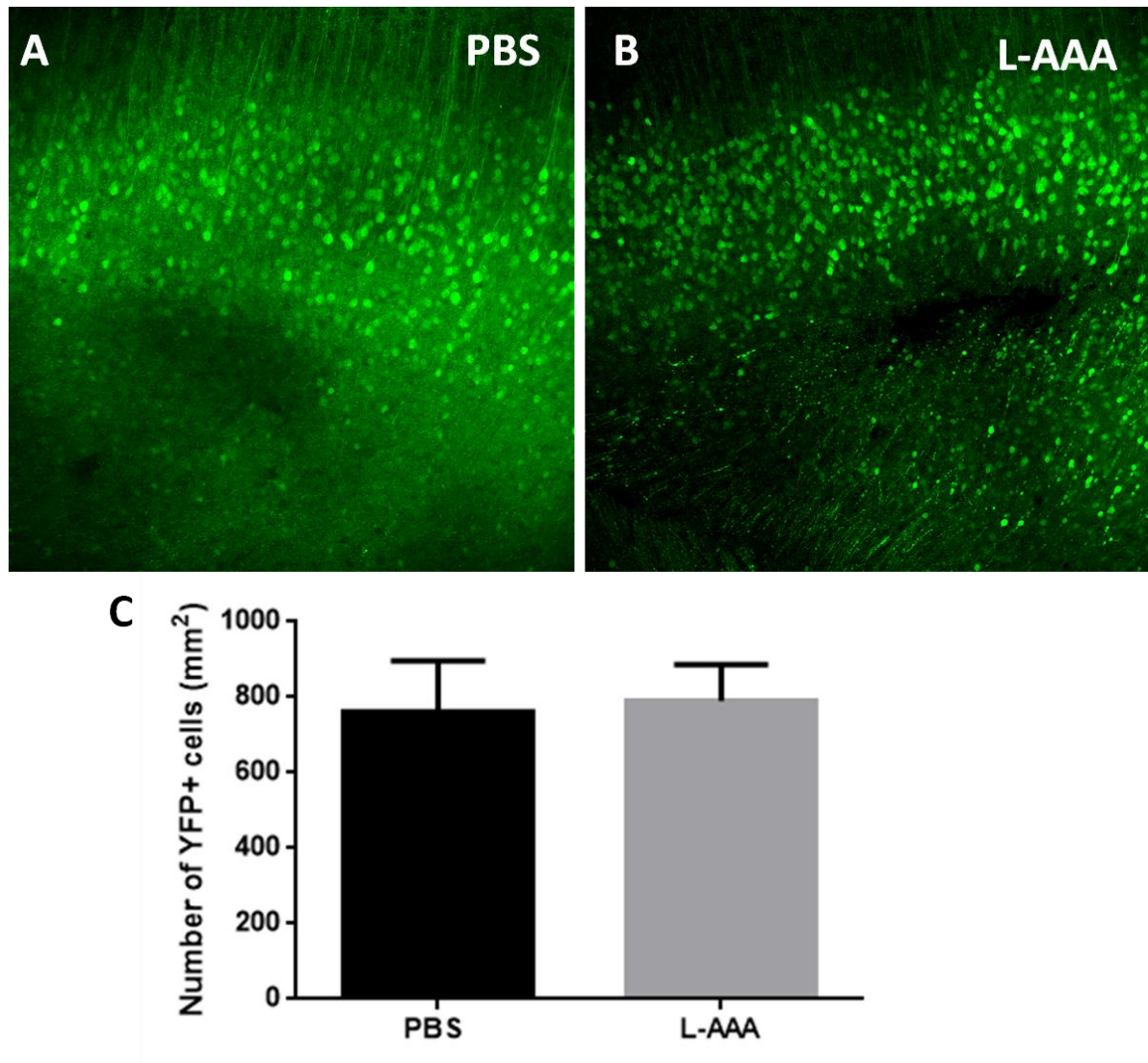


Figure 7.4: Effect of L-AAA on the viability of neurons

SLICK-H mice received one bilateral injection into the PLC (50µg/µl; 1µl). Representative pictures for PBS control group (A) and L-AAA (50µg/µl; 1µl) (B). L-AAA did not affect the number of YFP positive cells in the PLC 72 h post administration (C) Data are expressed as mean $\pm$ SEM, (PBS= 7 mice; L-AAA N= 8 mice).

4. Conclusion

The aim of this study was firstly to assess whether it was possible to assess neuronal complexity and spine density in the PLC of SLICK-H mice as previously described by Young et al. (2008). Despite the range of different techniques employed to improve the quality of the picture, analysis of neuronal morphology and spine density wasn't possible in the SLICK-H mice. As the neuronal cell bodies were easy to visualise, SLICK-H mice were used to quantify neuronal viability after L-AAA treatment. Although a high dose of L-AAA (20µg, 5µl, 1 injection) can decrease NeuN immunoreactivity (number of mature neurons) in the mPFC in rat (Lima et al., 2014), this study demonstrated that L-AAA, at the dose employed (50µg/µl), did not affect neuronal viability in SLICK-H mice when compared to controls.

Measuring spines was deemed unfeasible technically with the SLICK-H mice, hence the golgi-cox staining impregnation technique was adopted in subsequent experiments as described in chapter 5.

Appendix:

Publications and presentations

Publications

David, J.; Kebede, V.; McIntosh, A.L., O'Reilly, K, Thuery, G., Varidaki, A., Coffey, E., Harkin, A. Antidepressant-like properties of ZL006 and associated changes in dendritic spine density following delivery of the astrocytic toxin L-AAA to the PLC in mice (*in preparation*)

David, J.; O'Toole, E; O'Reilly, K; Assmann, N; Finlay, D; Harkin, A. Inhibitors of neuronal nitric oxide protect against neuronal atrophy and synapse loss provoked by L-alpha aminoadipic acid-treated astrocytes (*in preparation*).

O'Toole, E., **David, J.**, O'Reilly, K., Harkin, A. 'Peptide-mediated inhibition of the NOS1AP/nNOS interface protects against NMDA-induced atrophy in primary cortical neurons (*in preparation*)

Conference proceedings

David, J., O'Reilly, K., O'Toole, E., Harkin A. 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 – 39th All Ireland Schools of Pharmacy Conference, Cork, Ireland 2017 (*Presented by Kate O'Reilly*)

David, J., McIntosh, A.L., Harkin A. L α -Aminoadipic acid induced glial ablation in the Pre-Limbic cortex induces depressive and anxiety-like behaviour in mice – British Association for Psychopharmacology, Brighton, UK. 2016

David, J., McIntosh, A.L., Harkin A. L α -Aminoadipic acid provokes a reduction of astrocytes in the Pre-Limbic Cortex and depressive-like behaviour in mice –ECNP Workshop for Junior Scientists, Nice. France.2016

David, J., McIntosh, A.L., Harkin A. L-Alpha Aminoadipic Acid provokes a reduction in astroglial GFAP expression in the Pre-Limbic Cortex and induces anhedonia and depressive-like behaviours in mice – ICVS, Braga. Portugal. 2015

References

- Aan het Rot, M., Zarate, C.A., Charney, D.S., Mathew, S.J., 2012. Ketamine for Depression: Where Do We Go from Here? *Biol. Psychiatry* 72, 537–547. <https://doi.org/10.1016/j.biopsych.2012.05.003>
- Aarts, M., Liu, Y., Liu, L., Besshoh, S., Arundine, M., Gurd, J.W., Wang, Y.-T., Salter, M.W., Tymianski, M., 2002. Treatment of ischemic brain damage by perturbing NMDA receptor-PSD-95 protein interactions. *Science* 298, 846–850.
- Abbott, C.C., Jones, T., Lemke, N.T., Gallegos, P., McClintock, S.M., Mayer, A.R., Bustillo, J., Calhoun, V.D., 2014. Hippocampal structural and functional changes associated with electroconvulsive therapy response. *Transl. Psychiatry* 4, e483.
- Abdallah, C.G., Averill, L.A., Collins, K.A., Geha, P., Schwartz, J., Averill, C., DeWilde, K.E., Wong, E., Anticevic, A., Tang, C.Y., Iosifescu, D.V., Charney, D.S., Murrough, J.W., 2017. Ketamine Treatment and Global Brain Connectivity in Major Depression. *Neuropsychopharmacology* 42, 1210–1219.
- Abdallah, C.G., Sanacora, G., Duman, R.S., Krystal, J.H., 2015. Ketamine and rapid-acting antidepressants: a window into a new neurobiology for mood disorder therapeutics. *Annu. Rev. Med.* 66, 509–523.
- Acebes, A., Ferrús, A., 2000. Cellular and molecular features of axon collaterals and dendrites. *Trends Neurosci.* 23, 557–565.
- Aguado, F., Carmona, M.A., Pozas, E., Aguiló, A., Martínez-Guijarro, F.J., Alcantara, S., Borrell, V., Yuste, R., Ibañez, C.F., Soriano, E., 2003. BDNF regulates spontaneous correlated activity at early developmental stages by increasing synaptogenesis and expression of the K⁺/Cl⁻-co-transporter KCC2. *Development* 130, 1267–1280.
- Ahmari, S.E., Buchanan, J., Smith, S.J., 2000. Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat. Neurosci.* 3, 445.
- Akhtar, H., Bukhari, F., Nazir, M., Anwar, M.N., Shahzad, A., 2016. Therapeutic Efficacy of Neurostimulation for Depression: Techniques, Current Modalities, and Future Challenges. *Neurosci. Bull.* 32, 115–126. <https://doi.org/10.1007/s12264-015-0009-2>
- Alcaro, A., Panksepp, J., Wiczak, J., Hayes, D.J., Northoff, G., 2010. Is subcortical–cortical midline activity in depression mediated by glutamate and GABA? A cross-species translational approach. *Neurosci. Biobehav. Rev.* 34, 592–605.
- Allen, Nicola J., 2013. Role of glia in developmental synapse formation. *Curr. Opin. Neurobiol.* 23, 1027–1033.
- Amaral, M.D., Pozzo-Miller, L., 2007. BDNF induces calcium elevations associated with I_B BDNF, a nonselective cationic current mediated by TRPC channels. *J. Neurophysiol.* 98, 2476–2482.
- Anderson, R.M., Glanz, R.M., Johnson, S.B., Miller, M.M., Romig-Martin, S.A., Radley, J.J., 2016. Prolonged corticosterone exposure induces dendritic spine remodeling and attrition in the rat medial prefrontal cortex. *J. Comp. Neurol.* 524, 3729–3746. <https://doi.org/10.1002/cne.24027>
- Ansell, E.B., Rando, K., Tuit, K., Guarnaccia, J., Sinha, R., 2012. Cumulative Adversity and Smaller Gray Matter Volume in Medial Prefrontal, Anterior Cingulate, and Insula Regions.

Endocrinol. Epigenetics Extinction Early Life Traumatization 72, 57–64. <https://doi.org/10.1016/j.biopsych.2011.11.022>

Arenas, E., Trupp, M., Åkerud, P., Ibáñez, C.F., 1995. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* 15, 1465–1473.

Arikkath, J., 2012. Molecular mechanisms of dendrite morphogenesis. *Front. Cell. Neurosci.* 6.

Arnold, J.F., Zwiers, M.P., Fitzgerald, D.A., van Eijndhoven, P., Becker, E.S., Rinck, M., Fernández, G., Speckens, A.E., Tendolkar, I., 2012. Fronto-limbic microstructure and structural connectivity in remission from major depression. *Psychiatry Res. Neuroimaging* 204, 40–48.

Arnone, D., McIntosh, A.M., Ebmeier, K.P., Munafò, M.R., Anderson, I.M., 2012. Magnetic resonance imaging studies in unipolar depression: systematic review and meta-regression analyses. *Eur. Neuropsychopharmacol.* 22, 1–16.

Arnsten, A.F.T., 2009. Stress signalling pathways that impair prefrontal cortex structure and function. *Nat. Rev. Neurosci.* 10, 410–422. <https://doi.org/10.1038/nrn2648>

Arnsten, A.F.T., Raskind, M.A., Taylor, F.B., Connor, D.F., 2015. The effects of stress exposure on prefrontal cortex: Translating basic research into successful treatments for post-traumatic stress disorder. *Stress Resil.* 1, 89–99. <https://doi.org/10.1016/j.ynstr.2014.10.002>

Autry, A.E., Adachi, M., Nosyreva, E., Na, E.S., Los, M.F., Cheng, P., Kavalali, E.T., Monteggia, L.M., 2011. NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475, 91–95. <https://doi.org/10.1038/nature10130>

Bach, A., Pedersen, S.W., Dorr, L.A., Vallon, G., Ripoche, 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. *Sci. Rep.* 5.

Bakos, J., Bacova, Z., Grant, S.G., Castejon, A.M., Ostatnikova, D., 2015. Are molecules involved in neuritogenesis and axon guidance related to autism pathogenesis? *Neuromolecular Med.* 17, 297–304.

Baldwin, K.T., Eroglu, C., 2017. Molecular mechanisms of astrocyte-induced synaptogenesis. *Curr. Opin. Neurobiol.* 45, 113–120.

Banasr, M., Duman, R.S., 2008. Glial Loss in the Prefrontal Cortex Is Sufficient to Induce Depressive-like Behaviors. *Neurodegener. Dement. Depress.* 64, 863–870. <https://doi.org/10.1016/j.biopsych.2008.06.008>

Bandeira, F., Lent, R., Herculano-Houzel, S., 2009. Changing numbers of neuronal and non-neuronal cells underlie postnatal brain growth in the rat. *Proc. Natl. Acad. Sci.* 106, 14108–14113. <https://doi.org/10.1073/pnas.0804650106>

Banerjee, A., Castro, J., Sur, M., 2012. Rett syndrome: genes, synapses, circuits, and therapeutics. *Front. Psychiatry* 3.

Barnes, A.P., Polleux, F., 2009. Establishment of axon-dendrite polarity in developing neurons. *Annu. Rev. Neurosci.* 32.

Barres, B.A., 2008. The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron* 60, 430–440.

- Barria, A., Malinow, R., 2005. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron* 48, 289–301.
- Barria, A., Malinow, R., 2002. Subunit-specific NMDA receptor trafficking to synapses. *Neuron* 35, 345–353.
- Bastmeyer, M., O’Leary, D.D., 1996. Dynamics of target recognition by interstitial axon branching along developing cortical axons. *J. Neurosci.* 16, 1450–1459.
- Beattie, E.C., Stellwagen, D., Morishita, W., Bresnahan, J.C., Ha, B.K., Von Zastrow, M., Beattie, M.S., Malenka, R.C., 2002. Control of synaptic strength by glial TNF α . *Science* 295, 2282–2285.
- Bellamy, T.C., Griffiths, C., Garthwaite, J., 2002. Differential sensitivity of guanylyl cyclase and mitochondrial respiration to nitric oxide measured using clamped concentrations. *J. Biol. Chem.* 277, 31801–31807.
- Beller, J.A., Snow, D.M., 2014. Proteoglycans: road signs for neurite outgrowth. *Neural Regen. Res.* 9, 343.
- Bellon, A., Krebs, M.-O., Jay, T.M., 2011. Factoring neurotrophins into a neurite-based pathophysiological model of schizophrenia. *Prog. Neurobiol.* 94, 77–90.
- Belzung, C., Lemoine, M., 2011. Criteria of validity for animal models of psychiatric disorders: focus on anxiety disorders and depression. *Biol. Mood Anxiety Disord.* 1, 9.
- Berman, R.M., Cappiello, A., Anand, A., Oren, D.A., Heninger, G.R., Charney, D.S., Krystal, J.H., 2000. Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry* 47, 351–354. [https://doi.org/10.1016/S0006-3223\(99\)00230-9](https://doi.org/10.1016/S0006-3223(99)00230-9)
- Bernard, R., Kerman, I.A., Thompson, R.C., Jones, E.G., Bunney, W.E., Barchas, J.D., Schatzberg, A.F., Myers, R.M., Akil, H., Watson, S.J., 2010. Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Mol. Psychiatry* 16, 634.
- Berton, O., Hahn, C.-G., Thase, M.E., 2012. Are We Getting Closer to Valid Translational Models for Major Depression? *Science* 338, 75–79. <https://doi.org/10.1126/science.1222940>
- Berton, O., Nestler, E.J., 2006. New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7, 137–151. <https://doi.org/10.1038/nrn1846>
- Bessa, J.M., Ferreira, D., Melo, I., Marques, F., Cerqueira, J.J., Palha, J.A., Almeida, O.F.X., Sousa, N., 2008. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* 14, 764–773.
- Bi, F., Huang, C., Tong, J., Qiu, G., Huang, B., Wu, Q., Li, F., Xu, Z., Bowser, R., Xia, X.-G., 2013. Reactive astrocytes secrete lcn2 to promote neuron death. *Proc. Natl. Acad. Sci.* 110, 4069–4074.
- Bianchi, M., Hagan, J.J., Heidbreder, C.A., 2005. Neuronal plasticity, stress and depression: involvement of the cytoskeletal microtubular system? *Curr. DRUG TARGETS-CNS Neurol. Disord.* 4, 597.
- Biojone, C., Cabrera Casarotto, P., Regiane Joca, S., Castren, E., 2015. Interplay between nitric oxide and brain-derived neurotrophic factor in neuronal plasticity. *CNS Neurol. Disord.-Drug Targets Former. Curr. Drug Targets-CNS Neurol. Disord.* 14, 979–987.

- Blackmore, M.G., Moore, D.L., Smith, R.P., Goldberg, J.L., Bixby, J.L., Lemmon, V.P., 2010. High content screening of cortical neurons identifies novel regulators of axon growth. *Mol. Cell. Neurosci.* 44, 43–54. <https://doi.org/10.1016/j.mcn.2010.02.002>
- Bleakman, D., Lodge, D., 1998. Neuropharmacology of AMPA and kainate receptors. *Neuropharmacology* 37, 1187–1204. [https://doi.org/10.1016/S0028-3908\(98\)00139-7](https://doi.org/10.1016/S0028-3908(98)00139-7)
- Bliss, T.V., Lømo, T., 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* 232, 331–356.
- Bloss, E.B., Janssen, W.G., Ohm, D.T., Yuk, F.J., Wadsworth, S., Saardi, K.M., McEwen, B.S., Morrison, J.H., 2011. Evidence for Reduced Experience-Dependent Dendritic Spine Plasticity in the Aging Prefrontal Cortex. *J. Neurosci.* 31, 7831. <https://doi.org/10.1523/JNEUROSCI.0839-11.2011>
- Bock, J., Gruss, M., Becker, S., Braun, K., 2004. Experience-induced changes of dendritic spine densities in the prefrontal and sensory cortex: correlation with developmental time windows. *Cereb. Cortex* 15, 802–808.
- Bodea, L.-G., Wang, Y., Linnartz-Gerlach, B., Kopatz, J., Sinkkonen, L., Musgrove, R., Kaoma, T., Muller, A., Vallar, L., Di Monte, D.A., 2014. Neurodegeneration by activation of the microglial complement–phagosome pathway. *J. Neurosci.* 34, 8546–8556.
- Böhme, G.A., Bon, C., Stutzmann, J.-M., Doble, A., Blanchard, J.-C., 1991. Possible involvement of nitric oxide in long-term potentiation. *Eur. J. Pharmacol.* 199, 379–381. [https://doi.org/10.1016/0014-2999\(91\)90505-K](https://doi.org/10.1016/0014-2999(91)90505-K)
- Bora, E., Fornito, A., Pantelis, C., Yücel, M., 2012. Gray matter abnormalities in major depressive disorder: a meta-analysis of voxel based morphometry studies. *J. Affect. Disord.* 138, 9–18.
- Böttner, M., Harde, J., Barrenschee, M., Hellwig, I., Vogel, I., Ebsen, M., Wedel, T., 2013. GDNF induces synaptic vesicle markers in enteric neurons. *Neurosci. Res.* 77, 128–136.
- Bourgeron, T., 2009. A synaptic trek to autism. *Curr. Opin. Neurobiol.* 19, 231–234.
- Bourne, J., Harris, K.M., 2007. Do thin spines learn to be mushroom spines that remember? *Curr. Opin. Neurobiol.* <https://doi.org/10.1016/j.conb.2007.04.009>
- Bowley, M.P., Drevets, W.C., Öngür, D., Price, J.L., 2002. Low glial numbers in the amygdala in major depressive disorder. *Biol. Psychiatry* 52, 404–412. [https://doi.org/10.1016/S0006-3223\(02\)01404-X](https://doi.org/10.1016/S0006-3223(02)01404-X)
- Boyle, N.T., Connor, T.J., 2007. MDMA (“Ecstasy”) suppresses the innate IFN- γ response in vivo: a critical role for the anti-inflammatory cytokine IL-10. *Eur. J. Pharmacol.* 572, 228–238.
- Brancato, A., Bregman, D., Ahn, H.F., Pfau, M.L., Menard, C., Cannizzaro, C., Russo, S.J., Hodes, G.E., 2017. Sub-chronic variable stress induces sex-specific effects on glutamatergic synapses in the nucleus accumbens. *Neuroscience* 350, 180–189. <https://doi.org/10.1016/j.neuroscience.2017.03.014>
- Brenman, Jay E, Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C., Bredt, D.S., 1996. Interaction of Nitric Oxide Synthase with the Postsynaptic Density Protein PSD-95 and α 1-Syntrophin Mediated

by PDZ Domains. *Cell* 84, 757–767. [https://doi.org/10.1016/S0092-8674\(00\)81053-3](https://doi.org/10.1016/S0092-8674(00)81053-3)

Brenman, Jay E., Christopherson, K.S., Craven, S.E., McGee, A.W., Bredt, D.S., 1996. Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. *J. Neurosci.* 16, 7407–7415.

Broadbelt, K., Byne, W., Jones, L.B., 2002. Evidence for a decrease in basilar dendrites of pyramidal cells in schizophrenic medial prefrontal cortex. *Schizophr. Res.* 58, 75–81.

Brown, B.P., Kang, S.C., Gawelek, K., Zacharias, R.A., Anderson, S.R., Turner, C.P., Morris, J.K., 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, D.R., Kretschma, H.A., 1998. The gliotoxic mechanism of α -aminoadipic acid on cultured astrocytes. *J. Neurocytol.* 27, 109–118.

Buchser, W., Collins, M., Garyantes, T., Guha, R., Haney, S., Lemmon, V., Li, Z., Trask, O.J., 2004. Assay Development Guidelines for Image-Based High Content Screening, High Content Analysis and High Content Imaging, in: Sittampalam, G.S., Coussens, N.P., Brimacombe, K., Grossman, A., Arkin, M., Auld, D., Austin, C., Baell, J., Bejcek, B., Chung, T.D.Y., Dahlin, J.L., Devanaryan, V., Foley, T.L., Glicksman, M., Hall, M.D., Hass, J.V., Inglese, J., Iversen, P.W., Kahl, S.D., Kales, S.C., Lal-Nag, M., Li, Z., McGee, J., McManus, O., Riss, T., Trask, O.J., Weidner, J.R., Xia, M., Xu, X. (Eds.), *Assay Guidance Manual*. Eli Lilly & Company and the National Center for Advancing Translational Sciences, Bethesda (MD).

Buffo, A., Rolando, C., Ceruti, S., 2010. Astrocytes in the damaged brain: molecular and cellular insights into their reactive response and healing potential. *Biochem. Pharmacol.* 79, 77–89.

Burgdorf, J., Zhang, X. -l., Weiss, C., Gross, A., Boikess, S.R., Kroes, R.A., Khan, M.A., Burch, R.M., Rex, C.S., Disterhoft, J.F., Stanton, P.K., Moskal, J.R., 2015. The long-lasting antidepressant effects of rapastinel (GLYX-13) are associated with a metaplasticity process in the medial prefrontal cortex and hippocampus. *Neuroscience* 308, 202–211. <https://doi.org/10.1016/j.neuroscience.2015.09.004>

Bush, T.G., Puvanachandra, N., Horner, C.H., Polito, A., Ostenfeld, T., Svendsen, C.N., Mucke, L., Johnson, M.H., Sofroniew, M.V., 1999. Leukocyte Infiltration, Neuronal Degeneration, and Neurite Outgrowth after Ablation of Scar-Forming, Reactive Astrocytes in Adult Transgenic Mice. *Neuron* 23, 297–308. [https://doi.org/10.1016/S0896-6273\(00\)80781-3](https://doi.org/10.1016/S0896-6273(00)80781-3)

Bushong, E.A., Martone, M.E., Jones, Y.Z., Ellisman, M.H., 2002. Protoplasmic Astrocytes in CA1 Stratum Radiatum Occupy Separate Anatomical Domains. *J. Neurosci.* 22, 183–192.

Calabrese, F., Riva, M.A., Molteni, R., 2016. Synaptic alterations associated with depression and schizophrenia: potential as a therapeutic target. *Expert Opin. Ther. Targets* 20, 1195–1207.

Calabrese, V., Mancuso, C., Calvani, M., Rizzarelli, E., Butterfield, D.A., Stella, A.M.G., 2007. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nat. Rev. Neurosci.* 8, 766.

Campbell, S., MacQueen, G., 2004. The role of the hippocampus in the pathophysiology of

major depression. *J. Psychiatry Neurosci.*

Canossa, M., Giordano, E., Cappello, S., Guarnieri, C., Ferri, S., 2002. Nitric oxide down-regulates brain-derived neurotrophic factor secretion in cultured hippocampal neurons. *Proc. Natl. Acad. Sci.* 99, 3282–3287.

Cao, P., Maximov, A., Südhof, T.C., 2011. Activity-dependent IGF-1 exocytosis is controlled by the Ca²⁺-sensor synaptotagmin-10. *Cell* 145, 300–311.

Carey, L.M., Lee, W.-H., Gutierrez, T., Kulkarni, P.M., Thakur, G.A., Lai, Y.Y., Hohmann, A.G., 2017. Small molecule inhibitors of PSD95–nNOS protein–protein interactions suppress formalin-evoked Fos protein expression and nociceptive behavior in rats. *Neuroscience* 349, 303–317. <https://doi.org/10.1016/j.neuroscience.2017.02.055>

Carlson, S.W., Madathil, S.K., Sama, D.M., Gao, X., Chen, J., Saatman, K.E., 2014. Conditional overexpression of insulin-like growth factor-1 enhances hippocampal neurogenesis and restores immature neuron dendritic processes after traumatic brain injury. *J. Neuropathol. Exp. Neurol.* 73, 734–746.

Castillo-Gómez, E., Pérez-Rando, M., Bellés, M., Gilabert-Juan, J., Llorens, J.V., Carceller, H., Bueno-Fernández, C., García-Mompó, C., Ripoll-Martínez, B., Curto, Y., Sebastián-Ortega, N., Moltó, M.D., Sanjuan, J., Nacher, J., 2017. Early Social Isolation Stress and Perinatal NMDA Receptor Antagonist Treatment Induce Changes in the Structure and Neurochemistry of Inhibitory Neurons of the Adult Amygdala and Prefrontal Cortex. *eNeuro* 4, ENEURO.0034-17.2017. <https://doi.org/10.1523/ENEURO.0034-17.2017>

Castrén, E., Hen, R., 2013. Neuronal plasticity and antidepressant actions. *Trends Neurosci.* 36, 259–267.

Cerqueira, J.J., Mailliet, F., Almeida, O.F.X., Jay, T.M., Sousa, N., 2007. The Prefrontal Cortex as a Key Target of the Maladaptive Response to Stress. *J. Neurosci.* 27, 2781. <https://doi.org/10.1523/JNEUROSCI.4372-06.2007>

Chandley, M.J., Szebeni, K., Szebeni, A., Crawford, J., Stockmeier, C.A., Turecki, G., Miguel-Hidalgo, J.J., Ordway, G.A., 2013. Gene expression deficits in pontine locus coeruleus astrocytes in men with major depressive disorder. *J. Psychiatry Neurosci.* JPN 38, 276.

Chao, M.V., 2003. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 4, 299.

Chao, T.I., Rickmann, M., Wolff, J.R., 2002. The synapse-astrocyte boundary: an anatomical basis for an integrative role of glia in synaptic transmission. *Tripart. Synap. Glia Synaptic Transm.* 1, 3–23.

Charych, E.I., Akum, B.F., Goldberg, J.S., Jörnsten, R.J., Rongo, C., Zheng, J.Q., Firestein, B.L., 2006. Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95. *J. Neurosci.* 26, 10164–10176.

Cheetham, S.C., Crompton, M.R., Czudek, C., Horton, R.W., Katona, C.L., Reynolds, G.P., 1989. Serotonin concentrations and turnover in brains of depressed suicides. *Brain Res.* 502, 332–340.

Chen, C.-C., Huang, C.-C., Hsu, K.-S., 2016. Chronic Social Stress Affects Synaptic Maturation of Newly Generated Neurons in the Adult Mouse Dentate Gyrus. *Int. J. Neuropsychopharmacol.* 19, pyv097. <https://doi.org/10.1093/ijnp/pyv097>

- Chen, P.-S., Peng, G.-S., Li, G., Yang, S., Wu, X., Wang, C.-C., Wilson, B., Lu, R.-B., Gean, P.-W., Chuang, D.-M., Hong, J.-S., 2006. Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol Psychiatry* 11, 1116–1125.
- Chen, P.S., Peng, G.S., Li, G., Yang, S., Wu, X., Wang, C.C., Wilson, B., Lu, R.B., Gean, P.-W., Chuang, D.M., others, 2006. Valproate protects dopaminergic neurons in midbrain neuron/glia cultures by stimulating the release of neurotrophic factors from astrocytes. *Mol. Psychiatry* 11, 1116.
- Cheng, C., Lau, S.K., Doering, L.C., 2016. Astrocyte-secreted thrombospondin-1 modulates synapse and spine defects in the fragile X mouse model. *Mol. Brain* 9, 74.
- Choudary, P.V., Molnar, M., Evans, S.J., Tomita, H., Li, J., Vawter, M.P., Myers, R.M., Bunney, W.E., Akil, H., Watson, S.J., Jones, E.G., 2005. Altered cortical glutamatergic and GABAergic signal transmission with glial involvement in depression. *Proc. Natl. Acad. Sci. U. S. A.* 102, 15653–15658. <https://doi.org/10.1073/pnas.0507901102>
- Christoffel, D.J., Golden, S.A., Heshmati, M., Graham, A., Birnbaum, S., Neve, R.L., Hodes, G.E., Russo, S.J., 2012. Effects of Inhibitor of κ B Kinase Activity in the Nucleus Accumbens on Emotional Behavior. *Neuropsychopharmacology* 37, 2615–2623.
- Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mallowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., Barres, B.A., 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120, 421–433.
- Chung, K., Deisseroth, K., 2013. CLARITY for mapping the nervous system. *Nat. Methods* 10, 508–513. <https://doi.org/10.1038/nmeth.2481>
- Chung, W.-S., Clarke, L.E., Wang, G.X., Stafford, B.K., Sher, A., Chakraborty, C., Joung, J., Foo, L.C., Thompson, A., Chen, C., Smith, S.J., Barres, B.A., 2013. Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* 504, 394–400. <https://doi.org/10.1038/nature12776>
- Citri, A., Malenka, R.C., 2008. Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33, 18.
- Cline, H.T., 2001. Dendritic arbor development and synaptogenesis. *Curr. Opin. Neurobiol.* 11, 118–126.
- Cline, H.T., Constantine-Paton, M., 1990. NMDA receptor agonist and antagonists alter retinal ganglion cell arbor structure in the developing frog retinotectal projection. *J. Neurosci.* 10, 1197–1216.
- Cobb, J.A., O'Neill, K., Milner, J., Mahajan, G.J., Lawrence, T.J., May, W.L., Miguel-Hidalgo, J., Rajkowska, G., Stockmeier, C.A., 2016. Density of GFAP-immunoreactive astrocytes is decreased in left hippocampi in major depressive disorder. *Neuroscience* 316, 209–220. <https://doi.org/10.1016/j.neuroscience.2015.12.044>
- Cohen-Cory, S., 2002. The developing synapse: construction and modulation of synaptic structures and circuits. *Science* 298, 770–776.
- Cohen-Cory, S., Fraser, S.E., 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature* 378, 192.
- Cohen-Cory, S., Kidane, A.H., Shirkey, N.J., Marshak, S., 2010. Brain-derived neurotrophic

- factor and the development of structural neuronal connectivity. *Dev. Neurobiol.* 70, 271–288.
- Cole, J., Costafreda, S.G., McGuffin, P., Fu, C.H., 2011. Hippocampal atrophy in first episode depression: a meta-analysis of magnetic resonance imaging studies. *J. Affect. Disord.* 134, 483–487.
- Colón-Ramos, D.A., 2009. Synapse formation in developing neural circuits. *Curr. Top. Dev. Biol.* 87, 53–79.
- Cook, S.C., Wellman, C.L., 2004. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. *J Neurobiol* 60. <https://doi.org/10.1002/neu.20025>
- Copf, T., 2016. Impairments in dendrite morphogenesis as etiology for neurodevelopmental disorders and implications for therapeutic treatments. *Neurosci. Biobehav. Rev.* 68, 946–978.
- Cotter, D., Mackay, D., Landau, S., Kerwin, R., Everall, I., 2001. Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. *Arch. Gen. Psychiatry* 58, 545–553.
- Cotter, D., Wilson, S., Roberts, E., Kerwin, R., Everall, I.P., 2000. Increased dendritic MAP2 expression in the hippocampus in schizophrenia. *Schizophr. Res.* 41, 313–323.
- Cotter, D.R., Pariante, C.M., Everall, I.P., 2001. Glial cell abnormalities in major psychiatric disorders: the evidence and implications. *Neuropathol. Sev. Ment. Illn. Stud. Stanley Found. Neuropathol. Consort.* 55, 585–595. [https://doi.org/10.1016/S0361-9230\(01\)00527-5](https://doi.org/10.1016/S0361-9230(01)00527-5)
- Craig, A.M., Banker, G., 1994. Neuronal polarity. *Annu. Rev. Neurosci.* 17, 267–310.
- Crow, T.J., Cross, A.J., Cooper, S.J., Deakin, J.F.W., Ferrier, I.N., Johnson, J.A., Joseph, M.H., Owen, F., Poulter, M., Lofthouse, R., others, 1984. Neurotransmitter receptors and monoamine metabolites in the brains of patients with Alzheimer-type dementia and depression, and suicides. *Neuropharmacology* 23, 1561–1569.
- Cryan, J.F., Holmes, A., 2005. The ascent of mouse: advances in modelling human depression and anxiety. *Nat. Rev. Drug Discov.* 4, 775.
- Cryan, J.F., Mombereau, C., Vassout, A., 2005. The tail suspension test as a model for assessing antidepressant activity: Review of pharmacological and genetic studies in mice. *Anim. Models Depress. Antidepressant Act.* 29, 571–625. <https://doi.org/10.1016/j.neubiorev.2005.03.009>
- Czéh, B., Di Benedetto, B., 2013. Antidepressants act directly on astrocytes: Evidences and functional consequences. *Eur. Neuropsychopharmacol.* 23, 171–185. <https://doi.org/10.1016/j.euroneuro.2012.04.017>
- Czéh, B., Lucassen, P.J., 2007. What causes the hippocampal volume decrease in depression? *Eur. Arch. Psychiatry Clin. Neurosci.* 257, 250–260.
- Czeh, B., Simon, M., Schmelting, B., Hiemke, C., Fuchs, E., 2005. Astroglial Plasticity in the Hippocampus is Affected by Chronic Psychosocial Stress and Concomitant Fluoxetine Treatment. *Neuropsychopharmacology* 31, 1616–1626.
- Dailey, M.E., Smith, S.J., 1996. The dynamics of dendritic structure in developing hippocampal slices. *J. Neurosci.* 16, 2983–2994.
- Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S., Snyder, S.H., 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci.* 88,

6368–6371.

Day, J.S., O’neill, E., Cawley, C., Aretz, N.K., Kilroy, D., Gibney, S.M., Harkin, A., Connor, T.J., 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, C.A., Shering, A.F., Wokosin, D., Ilijic, E., Sun, Z., Sampson, A.R., others, 2006. Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat. Neurosci.* 9, 251.

De Keyser, J., Mostert, J.P., Koch, M.W., 2008. Dysfunctional astrocytes as key players in the pathogenesis of central nervous system disorders. *J. Neurol. Sci.* 267, 3–16. <https://doi.org/10.1016/j.jns.2007.08.044>

De Roo, M., Klauser, P., Mendez, P., Poglia, L., Muller, D., 2008. Activity-Dependent PSD Formation and Stabilization of Newly Formed Spines in Hippocampal Slice Cultures. *Cereb. Cortex* 18, 151–161. <https://doi.org/10.1093/cercor/bhm041>

Dean, B., Gray, L., Scarr, E., 2006. Regionally Specific Changes in Levels of Cortical S100 β in Bipolar 1 Disorder but not Schizophrenia. *Aust. N. Z. J. Psychiatry* 40, 217–224. <https://doi.org/10.1080/j.1440-1614.2006.01777.x>

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M., Anderson, D.J., 2006. The Transcription Factor NFIA Controls the Onset of Gliogenesis in the Developing Spinal Cord. *Neuron* 52, 953–968. <https://doi.org/10.1016/j.neuron.2006.11.019>

Dent, E.W., Gertler, F.B., 2003. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* 40, 209–227.

Dent, E.W., Gupton, S.L., Gertler, F.B., 2011. The growth cone cytoskeleton in axon outgrowth and guidance. *Cold Spring Harb. Perspect. Biol.* 3, a001800.

Deyama, S., Sugano, Y., Mori, S., Amano, T., Yoshioka, M., Kaneda, K., Minami, M., 2017. Activation of the NMDA receptor–neuronal nitric oxide synthase pathway within the ventral bed nucleus of the stria terminalis mediates the negative affective component of pain. *Neuropharmacology* 118, 59–68. <https://doi.org/10.1016/j.neuropharm.2017.03.008>

Dias-Ferreira, E., Sousa, J.C., Melo, I., Morgado, P., Mesquita, A.R., Cerqueira, J.J., Costa, R.M., Sousa, N., 2009. Chronic Stress Causes Frontostriatal Reorganization and Affects Decision-Making. *Science* 325, 621. <https://doi.org/10.1126/science.1171203>

Diazgranados, N., Ibrahim, L., Brutsche, N.E., Newberg, A., Kronstein, P., Khalife, S., Kammerer, W.A., Quezado, Z., Luckenbaugh, D.A., Salvatore, G., others, 2010. A randomized add-on trial of an N-methyl-D-aspartate antagonist in treatment-resistant bipolar depression. *Arch. Gen. Psychiatry* 67, 793–802.

Dijkstra, S., Bär, P.R., Gispen, W.H., Joosten, E.A.J., 1999. Selective stimulation of dendrite outgrowth from identified corticospinal neurons by homotopic astrocytes. *Neuroscience* 92, 1331–1342. [https://doi.org/10.1016/S0306-4522\(99\)00060-3](https://doi.org/10.1016/S0306-4522(99)00060-3)

Dingledine, R., Borges, K., Bowie, D., Traynelis, S.F., 1999. The Glutamate Receptor Ion Channels. *Pharmacol. Rev.* 51, 7.

Dong, C., Zhang, J., Yao, W., Ren, Q., Ma, M., Yang, C., Chaki, S., Hashimoto, K., 2017. Rapid and Sustained Antidepressant Action of the mGlu2/3 Receptor Antagonist MGS0039 in the Social Defeat Stress Model: Comparison with Ketamine. *Int. J.*

Neuropsychopharmacol. 20, 228–236. <https://doi.org/10.1093/ijnp/pyw089>

Dotti, C.G., Sullivan, C.A., Banker, G.A., 1988. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* 8, 1454–1468.

Doucet, M.V., Harkin, A., Dev, K.K., 2012. The PSD-95/nNOS complex: New drugs for depression? *Pharmacol. Ther.* 133, 218–229. <https://doi.org/10.1016/j.pharmthera.2011.11.005>

Doucet, M.V., Levine, H., Dev, K.K., Harkin, A., 2013. Small-Molecule Inhibitors at the PSD-95/nNOS Interface have Antidepressant-Like Properties in Mice. *Neuropsychopharmacology* 38, 1575–1584.

Doucet, M.V., 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. <https://doi.org/10.1016/j.neuroscience.2015.06.004>

Drevets, W.C., 2000. Functional anatomical abnormalities in limbic and prefrontal cortical structures in major depression. *Prog. Brain Res.* 126, 413–431.

Duman, C.H., Duman, R.S., 2015. Spine synapse remodeling in the pathophysiology and treatment of depression. *Neurosci. Lett.* 601, 20–29. <https://doi.org/10.1016/j.neulet.2015.01.022>

Duman, R.S., 2014. Neurobiology of stress, depression, and rapid acting antidepressants: remodeling synaptic connections. *Depress. Anxiety* 31, 291–296.

Duman, R.S., 2009. Neuronal damage and protection in the pathophysiology and treatment of psychiatric illness: stress and depression. *Dialogues Clin. Neurosci.* 11, 239.

Duman, Ronald S, Aghajanian, G.K., Sanacora, G., Krystal, J.H., 2016. Synaptic plasticity and depression: New insights from stress and rapid-acting antidepressants. *Nat. Med.* 22, 238–249. <https://doi.org/10.1038/nm.4050>

Duman, R.S., Li, N., 2012. A neurotrophic hypothesis of depression: role of synaptogenesis in the actions of NMDA receptor antagonists. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 2475. <https://doi.org/10.1098/rstb.2011.0357>

Dumont, A.O., Goursaud, S., Desmet, N., Hermans, E., 2014. Differential Regulation of Glutamate Transporter Subtypes by Pro-Inflammatory Cytokine TNF- α in Cortical Astrocytes from a Rat Model of Amyotrophic Lateral Sclerosis. *PLOS ONE* 9, e97649. <https://doi.org/10.1371/journal.pone.0097649>

Duric, V., Banasr, M., Stockmeier, C.A., Simen, A.A., Newton, S.S., Overholser, J.C., Jurjus, G.J., Dieter, L., Duman, R.S., 2013. Altered expression of synapse and glutamate related genes in post-mortem hippocampus of depressed subjects. *Int. J. Neuropsychopharmacol.* 16, 69–82.

Dzoljic, E., De Vries, R., Dzoljic, M., 1997. New and potent inhibitors of nitric oxide synthase reduce motor activity in mice. *Behav. Brain Res.* 87, 209–212. [https://doi.org/10.1016/S0166-4328\(97\)02281-X](https://doi.org/10.1016/S0166-4328(97)02281-X)

Efremova, L., Chovancova, P., Adam, M., Gutbier, S., Schildknecht, S., Leist, M., 2017. Switching from astrocytic neuroprotection to neurodegeneration by cytokine stimulation. *Arch. Toxicol.* 91, 231–246.

Eisendrath, S.J., Gillung, E., Delucchi, K.L., Segal, Z.V., Nelson, J.C., McInnes, L.A., Mathalon, D.H., Feldman, M.D., 2016. A Randomized Controlled Trial of Mindfulness-Based Cognitive Therapy for Treatment-Resistant Depression. *Psychother. Psychosom.* 85, 99–110.

Ernst, A.F., Gallo, G., Letourneau, P.C., McLoon, S.C., 2000. Stabilization of growing retinal axons by the combined signaling of nitric oxide and brain-derived neurotrophic factor. *J. Neurosci.* 20, 1458–1469.

Eroglu, C., Barres, B.A., 2010. Regulation of synaptic connectivity by glia. *Nature* 468, 223–231. <https://doi.org/10.1038/nature09612>

Esplugues, J.V., 2002. NO as a signalling molecule in the nervous system. *Br. J. Pharmacol.* 135, 1079–1095. <https://doi.org/10.1038/sj.bjp.0704569>

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. *Exp. Neurol.* 176, 41–54.

Falsig, J., Latta, M., Leist, M., 2004. Defined inflammatory states in astrocyte cultures: Correlation with susceptibility towards CD95-driven apoptosis. *J. Neurochem.* 88, 181–193.

Feldman, S., Conforti, N., Saphier, D., 1990. The preoptic area and bed nucleus of the stria terminalis are involved in the effects of the amygdala on adrenocortical secretion. *Neuroscience* 37, 775–779. [https://doi.org/10.1016/0306-4522\(90\)90107-F](https://doi.org/10.1016/0306-4522(90)90107-F)

Fenstermaker, V., Chen, Y., Ghosh, A., Yuste, R., 2004. Regulation of dendritic length and branching by semaphorin 3A. *Dev. Neurobiol.* 58, 403–412.

Fenton, E.Y., Fournier, N.M., Lussier, A.L., Romay-Tallon, R., Caruncho, H.J., Kalynchuk, L.E., 2015. Imipramine protects against the deleterious effects of chronic corticosterone on depression-like behavior, hippocampal reelin expression, and neuronal maturation. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 60, 52–59. <https://doi.org/10.1016/j.pnpbp.2015.02.001>

Ferrante, R.J., Kowall, N.W., Richardson, E.P., 1991. Proliferative and degenerative changes in striatal spiny neurons in Huntington's disease: a combined study using the section-Golgi method and calbindin D28k immunocytochemistry. *J. Neurosci.* 11, 3877–3887.

Ferrick, D.A., Neilson, A., Beeson, C., 2008. Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov. Today* 13, 268–274.

Fiala, J.C., Feinberg, M., Popov, V., Harris, K.M., 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J. Neurosci.* 18, 8900–8911.

Fiala, J.C., Spacek, J., Harris, K.M., 2002. Dendritic spine pathology: cause or consequence of neurological disorders? *Brain Res. Rev.* 39, 29–54.

Fifková, E., Delay, R.J., 1982. Cytoplasmic actin in neuronal processes as a possible mediator of synaptic plasticity. *J. Cell Biol.* 95, 345–350.

Fischer, M., Kaech, S., Knutti, D., Matus, A., 1998. Rapid actin-based plasticity in dendritic spines. *Neuron* 20, 847–854.

Florio, S., Loh, C., Huang, S., Iwamaye, A., Kitto, K., Fowler, K., Treiberg, J., Hayflick, J., Walker, J., Fairbanks, C., Lai, Y., 2009. Disruption of nNOS-PSD95 protein–protein

interaction inhibits acute thermal hyperalgesia and chronic mechanical allodynia in rodents. *Br. J. Pharmacol.* 158, 494–506. <https://doi.org/10.1111/j.1476-5381.2009.00300.x>

Flynn, K.C., 2013. The cytoskeleton and neurite initiation. *Bioarchitecture* 3, 86–109.

Foster, K.A., McLaughlin, N., Edbauer, D., Phillips, M., Bolton, A., Constantine-Paton, M., Sheng, M., 2010. Distinct roles of NR2A and NR2B cytoplasmic tails in long-term potentiation. *J. Neurosci.* 30, 2676–2685.

France, G., Fernández-Fernández, D., Burnell, E.S., Irvine, M.W., Monaghan, D.T., Jane, D.E., Bortolotto, Z.A., Collingridge, G.L., Volianskis, A., 2017. Multiple roles of GluN2B-containing NMDA receptors in synaptic plasticity in juvenile hippocampus. *Neuropharmacology* 112, 76–83.

Friedman, H.V., Bresler, T., Garner, C.C., Ziv, N.E., 2000. Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27, 57–69.

Frodl, T., Carballedo, A., Hughes, M.M., Saleh, K., Fagan, A., Skokauskas, N., McLoughlin, D.M., Meaney, J., O’keane, V., Connor, T.J., 2012. Reduced expression of glucocorticoid-inducible genes GILZ and SGK-1: high IL-6 levels are associated with reduced hippocampal volumes in major depressive disorder. *Transl. Psychiatry* 2, e88.

Frodl, T., Jäger, M., Smajstrlova, I., Born, C., Bottlender, R., Palladino, T., Reiser, M., Möller, H.-J., Meisenzahl, E.M., 2008. Effect of hippocampal and amygdala volumes on clinical outcomes in major depression: a 3-year prospective magnetic resonance imaging study. *J. Psychiatry Neurosci.* JPN 33, 423.

Gallardo, G., Barowski, J., Ravits, J., Siddique, T., Lingrel, J.B., Robertson, J., Steen, H., Bonni, A., 2014. An [alpha] 2-Na/K ATPase/[alpha]-adducin complex in astrocytes triggers non-cell autonomous neurodegeneration. *Nat. Neurosci.* 17, 1710–1719.

Gallo, G., 2006. RhoA-kinase coordinates F-actin organization and myosin II activity during semaphorin-3A-induced axon retraction. *J. Cell Sci.* 119, 3413–3423.

Gallo, G., Letourneau, P.C., 2000. Neurotrophins and the dynamic regulation of the neuronal cytoskeleton. *J. Neurobiol.* 44, 159–173.

Gallo, G., Letourneau, P.C., 1998. Localized sources of neurotrophins initiate axon collateral sprouting. *J. Neurosci.* 18, 5403–5414.

Gan, P., Ding, Z.-Y., Gan, C., Mao, R.-R., Zhou, H., Xu, L., Zhou, Q.-X., 2016. Corticosterone regulates fear memory via Rac1 activity in the hippocampus. *Psychoneuroendocrinology* 71, 86–93.

Gao, Y.-J., Ji, R.-R., 2010. Targeting astrocyte signaling for chronic pain. *Neurotherapeutics* 7, 482–493. <https://doi.org/10.1016/j.nurt.2010.05.016>

Garcia, L.S.B., Comim, C.M., Valvassori, S.S., Réus, G.Z., Stertz, L., Kapczinski, F., Gavioli, E.C., Quevedo, J., 2009. Ketamine treatment reverses behavioral and physiological alterations induced by chronic mild stress in rats. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 33, 450–455. <https://doi.org/10.1016/j.pnpbp.2009.01.004>

García-López, P., García-Marín, V., Freire, M., 2011. Dendritic spines and development: towards a unifying model of spinogenesis—a present day review of Cajal’s histological slides and drawings. *Neural Plast.* 2010.

- García-Rojo, G., Fresno, C., Vilches, N., Díaz-Véliz, G., Mora, S., Aguayo, F., Pacheco, A., Parra-Fiedler, N., Parra, C.S., Rojas, P.S., Tejos, M., Aliaga, E., Fiedler, J.L., 2017. The ROCK Inhibitor Fasudil Prevents Chronic Restraint Stress-Induced Depressive-Like Behaviors and Dendritic Spine Loss in Rat Hippocampus. *Int. J. Neuropsychopharmacol.* 20, 336–345. <https://doi.org/10.1093/ijnp/pyw108>
- Garey, L.J., Ong, W.Y., Patel, T.S., Kanani, M., Davis, A., Mortimer, A.M., Barnes, T.R.E., Hirsch, S.R., 1998. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. *J. Neurol. Neurosurg. Psychiatry* 65, 446–453.
- Garthwaite, J., Charles, S.L., Chess-Williams, R., 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336, 385–388.
- Garwood, C.J., Pooler, A.M., Atherton, J., Hanger, D.P., Noble, W., 2011. Astrocytes are important mediators of A β -induced neurotoxicity and tau phosphorylation in primary culture. *Cell Death Dis.* 2, e167.
- Geschwind, D.H., Levitt, P., 2007. Autism spectrum disorders: developmental disconnection syndromes. *Curr. Opin. Neurobiol.* 17, 103–111.
- Ghasemi, M., Phillips, C., Trillo, L., De Miguel, Z., Das, D., Salehi, A., 2014. The role of NMDA receptors in the pathophysiology and treatment of mood disorders. *Neurosci. Biobehav. Rev.* 47, 336–358. <https://doi.org/10.1016/j.neubiorev.2014.08.017>
- Gigliucci, V., Gormley, S., Gibney, S., Rouine, J., Kerskens, C., Connor, T.J., Harkin, A., 2014. Characterisation of the antidepressant properties of nitric oxide synthase inhibitors in the olfactory bulbectomised rat model of depression. *Eur. Neuropsychopharmacol.* 24, 1349–1361. <https://doi.org/10.1016/j.euroneuro.2014.05.003>
- Gilabert-Juan, J., Bueno-Fernandez, C., Castillo-Gomez, E., Nacher, J., 2017. Reduced interneuronal dendritic arborization in CA1 but not in CA3 region of mice subjected to chronic mild stress. *Brain Behav.* 7, e00534. <https://doi.org/10.1002/brb3.534>
- Gipson, C.D., Olive, M.F., 2017. Structural and functional plasticity of dendritic spines—root or result of behavior? *Genes Brain Behav.* 16, 101–117.
- Glantz, L.A., Lewis, D.A., 2000. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch. Gen. Psychiatry* 57, 65–73.
- Goldberg, J.L., 2003. How does an axon grow? *Genes Dev.* 17, 941–958.
- Golden, S.A., Christoffel, D.J., Heshmati, M., Hodes, G.E., Magida, J., Davis, K., Cahill, M.E., Dias, C., Ribeiro, E., Ables, J.L., 2013. Epigenetic regulation of RAC1 induces synaptic remodeling in stress disorders and depression. *Nat. Med.* 19, 337.
- Gomez, T.M., Letourneau, P.C., 2014. Actin dynamics in growth cone motility and navigation. *J. Neurochem.* 129, 221–234.
- Gonzalez, A., Moya-Alvarado, G., Gonzalez-Billaut, C., Bronfman, F.C., 2016. Cellular and molecular mechanisms regulating neuronal growth by brain-derived neurotrophic factor. *Cytoskeleton* 73, 612–628.
- Goodman, C.S., 1996. Mechanisms and molecules that control growth cone guidance. *Annu. Rev. Neurosci.* 19, 341–377.

- Gordon-Weeks, P.R., Fournier, A.E., 2014. Neuronal cytoskeleton in synaptic plasticity and regeneration. *J. Neurochem.* 129, 206–212.
- Gos, T., Bock, J., Poeggel, G., Braun, K., 2008. Stress-induced synaptic changes in the rat anterior cingulate cortex are dependent on endocrine developmental time windows. *Synapse* 62, 229–232.
- Gos, T., Schroeter, M.L., Lessel, W., Bernstein, H.-G., Dobrowolny, H., Schiltz, K., Bogerts, B., Steiner, J., 2013. S100B-immunopositive astrocytes and oligodendrocytes in the hippocampus are differentially afflicted in unipolar and bipolar depression: A postmortem study. *J. Psychiatr. Res.* 47, 1694–1699. <https://doi.org/10.1016/j.jpsychires.2013.07.005>
- Gosselin, R.-D., Gibney, S., O'Malley, D., Dinan, T.G., Cryan, J.F., 2009. Region specific decrease in glial fibrillary acidic protein immunoreactivity in the brain of a rat model of depression. *Neuroscience* 159, 915–925. <https://doi.org/10.1016/j.neuroscience.2008.10.018>
- Gourley, S.L., Swanson, A.M., Koleske, A.J., 2013. Corticosteroid-Induced Neural Remodeling Predicts Behavioral Vulnerability and Resilience. *J. Neurosci.* 33, 3107. <https://doi.org/10.1523/JNEUROSCI.2138-12.2013>
- Grabe, H.J., Ahrens, N., Rose, H.-J., Kessler, C., Freyberger, H.J., 2001. Neurotrophic Factor S100beta in Major Depression. *Neuropsychobiology* 44, 88–90.
- Graf, E.R., Zhang, X., Jin, S.-X., Linhoff, M.W., Craig, A.M., 2004. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119, 1013–1026.
- Graveland, G.A., Williams, R.S., DiFiglia, M., 1985. Evidence for degenerative and regenerative changes in neostriatal spiny neurons in Huntington's disease. *Science* 227, 770–773.
- Gray, G.E., Sanes, J.R., 1991. Migratory paths and phenotypic choices of clonally related cells in the avian optic tectum. *Neuron* 6, 211–225. [https://doi.org/10.1016/0896-6273\(91\)90357-6](https://doi.org/10.1016/0896-6273(91)90357-6)
- Gusel'nikova, V.V., Korzhevskiy, D.E., 2015. NeuN as a neuronal nuclear antigen and neuron differentiation marker. *Acta Naturae Англоязычная Версия* 7.
- Gutierrez, H., Davies, A.M., 2007. A fast and accurate procedure for deriving the Sholl profile in quantitative studies of neuronal morphology. *J. Neurosci. Methods* 163, 24–30.
- Haga, K.K., Gregory, L.J., Hicks, C.A., Ward, M.A., Beech, J.S., Bath, P.W., Williams, S.C., O'Neill, M.J., 2003. The neuronal nitric oxide synthase inhibitor, TRIM, as a neuroprotective agent: effects in models of cerebral ischaemia using histological and magnetic resonance imaging techniques. *Brain Res.* 993, 42–53. <https://doi.org/10.1016/j.brainres.2003.08.063>
- Hajduková, L., Sobek, O., Prchalová, D., Bílková, Z., Koudelková, M., Lukášková, J., Matuchová, I., 2015. Biomarkers of Brain Damage: S100B and NSE Concentrations in Cerebrospinal Fluid—A Normative Study. *BioMed Res. Int.* 2015, 379071. <https://doi.org/10.1155/2015/379071>
- Hajszan, T., Dow, A., Warner-Schmidt, J.L., Szigeti-Buck, K., Sallam, N.L., Parducz, A., Leranth, C., Duman, R.S., 2009. Remodeling of Hippocampal Spine Synapses in the Rat Learned Helplessness Model of Depression. *Trauma Neurodev. Neuroplast. Implic. Later Anxiety* 65, 392–400. <https://doi.org/10.1016/j.biopsych.2008.09.031>

- Hajszan, T., MacLusky, N.J., Leranath, C., 2005. Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. *Eur. J. Neurosci.* 21, 1299–1303. <https://doi.org/10.1111/j.1460-9568.2005.03968.x>
- Hall, A., Lalli, G., 2010. Rho and Ras GTPases in axon growth, guidance, and branching. *Cold Spring Harb. Perspect. Biol.* 2, a001818.
- Halliday, G.M., Holton, J.L., Revesz, T., Dickson, D.W., 2011. Neuropathology underlying clinical variability in patients with synucleinopathies. *Acta Neuropathol. (Berl.)* 122, 187–204. <https://doi.org/10.1007/s00401-011-0852-9>
- Hamani, C., Diwan, M., Macedo, C.E., Brandão, M.L., Shumake, J., Gonzalez-Lima, F., Raymond, R., Lozano, A.M., Fletcher, P.J., Nobrega, J.N., 2010. Antidepressant-like effects of medial prefrontal cortex deep brain stimulation in rats. *Biol. Psychiatry* 67, 117–124. <https://doi.org/10.1016/j.biopsych.2009.08.025>
- Hamilton, J.P., Siemer, M., Gotlib, I.H., 2008. Amygdala volume in major depressive disorder: a meta-analysis of magnetic resonance imaging studies. *Mol. Psychiatry* 13, 993.
- Han, Q., Cai, T., Tagle, D.A., Robinson, H., Li, J., 2008. Substrate specificity and structure of human aminoadipate aminotransferase/kynurenine aminotransferase II. *Biosci. Rep.* 28, 205–215.
- Handy, R.L.C., Moore, P.K., 1998. A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. *Br. J. Pharmacol.* 123, 1119–1126. <https://doi.org/10.1038/sj.bjp.0701735>
- Hansen, S.M., Berezin, V., Bock, E., 2008. Signaling mechanisms of neurite outgrowth induced by the cell adhesion molecules NCAM and N-cadherin. *Cell. Mol. Life Sci.* 65, 3809–3821.
- Hansson, E., Muyderman, H., Leonova, J., Allansson, L., Sinclair, J., Blomstrand, F., Thorlin, T., Nilsson, M., Rönnbäck, L., 2000. Astroglia and glutamate in physiology and pathology: aspects on glutamate transport, glutamate-induced cell swelling and gap-junction communication. *Neurochem. Int.* 37, 317–329. [https://doi.org/10.1016/S0197-0186\(00\)00033-4](https://doi.org/10.1016/S0197-0186(00)00033-4)
- Hardingham, G.E., Bading, H., 2010. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci* 11, 682–696. <https://doi.org/10.1038/nrn2911>
- Hardingham, N., Dachtler, J., Fox, K., 2013. The role of nitric oxide in pre-synaptic plasticity and homeostasis. *Front. Cell. Neurosci.* 7.
- Harkin, A., Houlihan, D.D., Kelly, J.P., 2002. Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine. *J. Psychopharmacol. (Oxf.)* 16, 115–123.
- Harrison, D.C., Medhurst, A.D., Bond, B.C., Campbell, C.A., Davis, R.P., Philpott, K.L., 2000. The use of quantitative RT-PCR to measure mRNA expression in a rat model of focal ischemia—caspase-3 as a case study. *Mol. Brain Res.* 75, 143–149.
- Harrison, P.J., 2004. The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. *Psychopharmacology (Berl.)* 174, 151–162.
- Harvey, I., Ron, M.A., Du Boulay, G., Wicks, D., Lewis, S.W., Murray, R.M., 1993.

- Reduction of cortical volume in schizophrenia on magnetic resonance imaging. *Psychol. Med.* 23, 591–604.
- Hasler, G., Northoff, G., 2011. Discovering imaging endophenotypes for major depression. *Mol. Psychiatry* 16, 604.
- Hasler, G., van der Veen, J.W., Tumonis, T., Meyers, N., Shen, J., Drevets, W.C., 2007. Reduced prefrontal glutamate/glutamine and γ -aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. *Arch. Gen. Psychiatry* 64, 193–200.
- Häusser, M., Spruston, N., Stuart, G.J., 2000. Diversity and dynamics of dendritic signaling. *Science* 290, 739–744.
- Haydon, P.G., 2001. Glia: listening and talking to the synapse. *Nat Rev Neurosci* 2, 185–193. <https://doi.org/10.1038/35058528>
- He, C.X., Portera-Cailliau, C., 2013. The trouble with spines in fragile X syndrome: density, maturity and plasticity. *Neuroscience* 251, 120–128.
- He, P., Shen, Y., 2009. Interruption of β -catenin signaling reduces neurogenesis in Alzheimer's disease. *J. Neurosci.* 29, 6545–6557.
- Hering, H., Sheng, M., 2001. Dendritic spines: structure, dynamics and regulation. *Nat Rev Neurosci* 2, 880–888. <https://doi.org/10.1038/35104061>
- Hermes, J., Dorostkar, M.M., 2016. Dendritic spine pathology in neurodegenerative diseases. *Annu. Rev. Pathol. Mech. Dis.* 11, 221–250.
- Holtzheimer, P.E., Mayberg, H.S., 2011a. Stuck in a Rut: Rethinking Depression and its Treatment. *Trends Neurosci.* 34, 1–9. <https://doi.org/10.1016/j.tins.2010.10.004>
- Holtzheimer, P.E., Mayberg, H.S., 2011b. Deep Brain Stimulation for Psychiatric Disorders. *Annu. Rev. Neurosci.* 34, 289–307. <https://doi.org/10.1146/annurev-neuro-061010-113638>
- Homayoun, H., Moghaddam, B., 2007. NMDA Receptor Hypofunction Produces Opposite Effects on Prefrontal Cortex Interneurons and Pyramidal Neurons. *J. Neurosci.* 27, 11496. <https://doi.org/10.1523/JNEUROSCI.2213-07.2007>
- Horch, H.W., Katz, L.C., 2002. BDNF release from single cells elicits local dendritic growth in nearby neurons. *Nat. Neurosci.* 5, 1177.
- 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. *Neurochem. Int.* 79, 57–64. <https://doi.org/10.1016/j.neuint.2014.10.005>
- Hu, X., Yuan, Y., Wang, D., Su, Z., 2016. Heterogeneous astrocytes: Active players in CNS. *Brain Res. Bull.* 125, 1–18. <https://doi.org/10.1016/j.brainresbull.2016.03.017>
- Huck, S., Grass, F., Hortnagl, H., 1984. The glutamate analogue alpha-amino adipic acid is taken up by astrocytes before exerting its gliotoxic effect in vitro. *J. Neurosci.* 4, 2650–2657.
- Hughes, E.G., Maguire, J.L., McMinin, M.T., Scholz, R.E., Sutherland, M.L., 2004. Loss of glial fibrillary acidic protein results in decreased glutamate transport and inhibition of PKA-induced EAAT2 cell surface trafficking. *Mol. Brain Res.* 124, 114–123. <https://doi.org/10.1016/j.molbrainres.2004.02.021>

- Hutsler, J.J., Zhang, H., 2010. Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain Res.* 1309, 83–94.
- Ibrahim, L., DiazGranados, N., Franco-Chaves, J., Brutsche, N., Henter, I.D., Kronstein, P., Moaddel, R., Wainer, I., Luckenbaugh, D.A., Manji, H.K., Zarate, C.A., Jr, 2012. Course of Improvement in Depressive Symptoms to a Single Intravenous Infusion of Ketamine vs Add-on Riluzole: Results from a 4-Week, Double-Blind, Placebo-Controlled Study. *Neuropsychopharmacology* 37, 1526–1533.
- Ikonomidou, C., Turski, L., 2002. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol.* 1, 383–386. [https://doi.org/10.1016/S1474-4422\(02\)00164-3](https://doi.org/10.1016/S1474-4422(02)00164-3)
- Imai, Y., Ibata, I., Ito, D., Ohsawa, K., Kohsaka, S., 1996. A novel gene in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem. Biophys. Res. Commun.* 224, 855–862.
- Ippolito, D.M., Eroglu, C., 2010. Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. *J. Vis. Exp. JoVE*.
- Irwin, S.A., Galvez, R., Greenough, W.T., 2000. Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cereb. Cortex* 10, 1038–1044.
- Jacobsen, C.T., Miller, R.H., 2003. Control of Astrocyte Migration in the Developing Cerebral Cortex. *Dev. Neurosci.* 25, 207–216.
- Jayakumar, A.R., Tong, X.Y., Curtis, K.M., Ruiz-Cordero, R., Shamaladevi, N., Abuzamel, M., Johnstone, J., Gaidosh, G., Rama Rao, K.V., Norenberg, M.D., 2014. Decreased astrocytic thrombospondin-1 secretion after chronic ammonia treatment reduces the level of synaptic proteins: in vitro and in vivo studies. *J. Neurochem.* 131, 333–347.
- Jiang, D., Liang, J., Noble, P.W., 2007. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol* 23, 435–461.
- Jin, K., Mao, X.O., Greenberg, D.A., 2006. Vascular endothelial growth factor stimulates neurite outgrowth from cerebral cortical neurons via Rho kinase signaling. *Dev. Neurobiol.* 66, 236–242.
- Johansson, S., Strömberg, I., 2002. Guidance of dopaminergic neuritic growth by immature astrocytes in organotypic cultures of rat fetal ventral mesencephalon. *J. Comp. Neurol.* 443, 237–249. <https://doi.org/10.1002/cne.10119>
- Johnson, S.A., Wang, J.-F., Sun, X., McEwen, B.S., Chattarji, S., Young, L.T., 2009. Lithium treatment prevents stress-induced dendritic remodeling in the rodent amygdala. *Neuroscience* 163, 34–39. <https://doi.org/10.1016/j.neuroscience.2009.06.005>
- Jüttner, R., Rathjen, F.G., 2005. Molecular analysis of axonal target specificity and synapse formation. *Cell. Mol. Life Sci.* 62, 2811–2827.
- Kalueff, A.V., Stewart, A.M., Song, C., Berridge, K.C., Graybiel, A.M., Fentress, J.C., 2016. Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nat Rev Neurosci* 17, 45–59.
- Kalus, P., Muller, T.J., Zuschratter, W., Senitz, D., 2000. The dendritic architecture of prefrontal pyramidal neurons in schizophrenic patients. *Neuroreport* 11, 3621–3625.

- Kamiguchi, H., Lemmon, V., 2000. Recycling of the cell adhesion molecule L1 in axonal growth cones. *J. Neurosci.* 20, 3676–3686.
- Kang, H.J., Voleti, B., Hajszan, T., Rajkowska, G., Stockmeier, C.A., Licznarski, P., Lepack, A., Majik, M.S., Jeong, L.S., Banasr, M., others, 2012. Decreased expression of synapse-related genes and loss of synapses in major depressive disorder. *Nat. Med.* 18, 1413–1417.
- Kasai, H., Fukuda, M., Watanabe, S., Hayashi-Takagi, A., Noguchi, J., 2010. Structural dynamics of dendritic spines in memory and cognition. *Trends Neurosci.* 33, 121–129.
- Kasai, H., Matsuzaki, M., Noguchi, J., Yasumatsu, N., Nakahara, H., 2003. Structure–stability–function relationships of dendritic spines. *Trends Neurosci.* 26, 360–368.
- Kassem, M.S., Lagopoulos, J., Stait-Gardner, T., Price, W.S., Chohan, T.W., Arnold, J.C., Hatton, S.N., Bennett, M.R., 2013. Stress-Induced Grey Matter Loss Determined by MRI Is Primarily Due to Loss of Dendrites and Their Synapses. *Mol. Neurobiol.* 47, 645–661. <https://doi.org/10.1007/s12035-012-8365-7>
- Kawano, H., Kimura-Kuroda, J., Komuta, Y., Yoshioka, N., Li, H.P., Kawamura, K., Li, Y., Raisman, G., 2012. Role of the lesion scar in the response to damage and repair of the central nervous system. *Cell Tissue Res.* 349, 169–180. <https://doi.org/10.1007/s00441-012-1336-5>
- Kemp, J.A., McKernan, R.M., 2002. NMDA receptor pathways as drug targets. *Nat Neurosci.*
- Kempton, M.J., Salvador, Z., Munafò, M.R., Geddes, J.R., Simmons, A., Frangou, S., Williams, S.C., 2011. Structural neuroimaging studies in major depressive disorder: meta-analysis and comparison with bipolar disorder. *Arch. Gen. Psychiatry* 68, 675–690.
- Keshavan, M.S., Anderson, S., Pettergrew, J.W., 1994. Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? The Feinberg hypothesis revisited. *J. Psychiatr. Res.* 28, 239–265.
- Kessler, R.C., 1997. THE EFFECTS OF STRESSFUL LIFE EVENTS ON DEPRESSION. *Annu. Rev. Psychol.* 48, 191–214. <https://doi.org/10.1146/annurev.psych.48.1.191>
- Kew, J.N.C., Kemp, J.A., 2005. Ionotropic and metabotropic glutamate receptor structure and pharmacology. *Psychopharmacology (Berl.)* 179, 4–29. <https://doi.org/10.1007/s00213-005-2200-z>
- Khodosevich, K., Monyer, H., 2010. Signaling involved in neurite outgrowth of postnatally born subventricular zone neurons in vitro. *BMC Neurosci.* 11, 18.
- Kim, D.-M., Leem, Y.-H., 2016. Chronic stress-induced memory deficits are reversed by regular exercise via AMPK-mediated BDNF induction. *Neuroscience* 324, 271–285. <https://doi.org/10.1016/j.neuroscience.2016.03.019>
- Kim, P., Evans, G.W., Angstadt, M., Ho, S.S., Sripada, C.S., Swain, J.E., Liberzon, I., Phan, K.L., 2013. Effects of childhood poverty and chronic stress on emotion regulatory brain function in adulthood. *Proc. Natl. Acad. Sci.* 110, 18442. <https://doi.org/10.1073/pnas.1308240110>
- Kiryushko, D., Berezin, V., Bock, E., 2004. Regulators of neurite outgrowth: role of cell adhesion molecules. *Ann. N. Y. Acad. Sci.* 1014, 140–154.
- Klempan, T.A., Sequeira, A., Canetti, L., Lalovic, A., Ernst, C., French-Mullen, J., Turecki,

G., 2007. Altered expression of genes involved in ATP biosynthesis and GABAergic neurotransmission in the ventral prefrontal cortex of suicides with and without major depression. *Mol. Psychiatry* 14, 175.

Klöcker, N., Cellerino, A., Bähr, M., 1998. Free radical scavenging and inhibition of nitric oxide synthase potentiates the neurotrophic effects of brain-derived neurotrophic factor on axotomized retinal ganglion cells in vivo. *J. Neurosci.* 18, 1038–1046.

Koehler, R.C., Roman, R.J., Harder, D.R., 2009. Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci.* 32, 160–169. <https://doi.org/10.1016/j.tins.2008.11.005>

Koh, D.S., Burnashev, N., Jonas, P., 1995. Block of native Ca (2+)-permeable AMPA receptors in rat brain by intracellular polyamines generates double rectification. *J. Physiol.* 486, 305–312.

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. *Behav. Brain Res.* 224, 107–111. <https://doi.org/10.1016/j.bbr.2011.05.035>

Kolarow, R., Kuhlmann, C.R., Munsch, T., Zehendner, C., Brigadski, T., Luhmann, H.J., Lessmann, V., 2014. BDNF-induced nitric oxide signals in cultured rat hippocampal neurons: time course, mechanism of generation, and effect on neurotrophin secretion. *Front. Cell. Neurosci.* 8.

Kolb, B., Whishaw, I.Q., 1998. Brain plasticity and behavior. *Annu. Rev. Psychol.* 49, 43–64.

Koles, K., Budnik, V., 2012. Wnt signaling in neuromuscular junction development. *Cold Spring Harb. Perspect. Biol.* 4, a008045.

Kolomeets, N.S., Orlovskaya, D.D., Rachmanova, V.I., Uranova, N.A., 2005. Ultrastructural alterations in hippocampal mossy fiber synapses in schizophrenia: a postmortem morphometric study. *Synapse* 57, 47–55.

Kolomeets, N.S., Orlovskaya, D.D., Uranova, N.A., 2007. Decreased numerical density of CA3 hippocampal mossy fiber synapses in schizophrenia. *Synapse* 61, 615–621.

Konradi, C., Heckers, S., 2003. Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. *Pharmacol. Ther.* 97, 153–179. [https://doi.org/10.1016/S0163-7258\(02\)00328-5](https://doi.org/10.1016/S0163-7258(02)00328-5)

Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A., Moryś, J., 2017. BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. *Cell. Mol. Neurobiol.*

Kriegelstein, K., Zheng, F., Unsicker, K., Alzheimer, C., 2011. More than being protective: functional roles for TGF- β /activin signaling pathways at central synapses. *Trends Neurosci.* 34, 421–429.

Küçükibrahimoğlu, E., Saygın, M.Z., Çalışkan, M., Kaplan, O.K., Ünsal, C., Gören, M.Z., 2009. The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression. *Eur. J. Clin. Pharmacol.* 65, 571–577. <https://doi.org/10.1007/s00228-009-0650-7>

Kugaya, A., Sanacora, G., 2005. Beyond Monoamines: Glutamatergic Function in Mood Disorders. *CNS Spectr.* 10, 808–819. <https://doi.org/10.1017/S1092852900010403>

- Kulkarni, V.A., Firestein, B.L., 2012. The dendritic tree and brain disorders. *Mol. Cell. Neurosci.* 50, 10–20.
- Kumar, V., Zhang, M.-X., Swank, M.W., Kunz, J., Wu, G.-Y., 2005. Regulation of dendritic morphogenesis by Ras–PI3K–Akt–mTOR and Ras–MAPK signaling pathways. *J. Neurosci.* 25, 11288–11299.
- Kuo, T.-Y., Chen, C.-Y., Hsueh, Y.-P., 2010. Bcl11A/CTIP1 mediates the effect of the glutamate receptor on axon branching and dendrite outgrowth. *J. Neurochem.* 114, 1381–1392.
- Kurt, M.A., Kafa, M.I., Dierssen, M., Davies, D.C., 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 Res.* 1022, 101–109.
- Lai, C.-H., 2013. Gray matter volume in major depressive disorder: a meta-analysis of voxel-based morphometry studies. *Psychiatry Res. Neuroimaging* 211, 37–46.
- Lai, K.-O., Ip, N.Y., 2013. Structural plasticity of dendritic spines: the underlying mechanisms and its dysregulation in brain disorders. *Biochim. Biophys. Acta BBA-Mol. Basis Dis.* 1832, 2257–2263.
- Landis, D.M., Reese, T.S., 1983. Cytoplasmic organization in cerebellar dendritic spines. *J. Cell Biol.* 97, 1169–1178.
- Lau, T., Bigio, B., Zelli, D., McEwen, B., Nasca, C., 2017. Stress-induced structural plasticity of medial amygdala stellate neurons and rapid prevention by a candidate antidepressant. *Mol. Psychiatry* 22, 227–234. <https://doi.org/10.1038/mp.2016.68>
- Lauritsen, C., Mazuera, S., Lipton, R.B., Ashina, S., 2016. Intravenous ketamine for subacute treatment of refractory chronic migraine: a case series. *J. Headache Pain* 17, 106. <https://doi.org/10.1186/s10194-016-0700-3>
- Lavezzari, G., McCallum, J., Dewey, C.M., Roche, K.W., 2004. Subunit-specific regulation of NMDA receptor endocytosis. *J. Neurosci.* 24, 6383–6391.
- Ledda, F., Paratcha, G., Sandoval-Guzmán, T., Ibáñez, C.F., 2007. GDNF and GFR [alpha] 1 promote formation of neuronal synapses by ligand-induced cell adhesion. *Nat. Neurosci.* 10, 293.
- Lee, W.-H., Xu, Z., Ashpole, N.M., Hudmon, A., Kulkarni, P.M., Thakur, G.A., Lai, Y.Y., Hohmann, A.G., 2015. Small molecule inhibitors of PSD95-nNOS protein-protein interactions as novel analgesics. *Neuropharmacology* 97, 464–475. <https://doi.org/10.1016/j.neuropharm.2015.05.038>
- Lestanova, Z., Bacova, Z., Bakos, J., 2016. Mechanisms involved in the regulation of neuropeptide-mediated neurite outgrowth: a minireview. *Endocr. Regul.* 50, 72–82.
- Levinson, J.N., El-Husseini, A., 2005. Building excitatory and inhibitory synapses: balancing neuroligin partnerships. *Neuron* 48, 171–174.
- Li, A.-J., Suzuki, S., Suzuki, M., Mizukoshi, E., Imamura, T., 2002. Fibroblast growth factor-2 increases functional excitatory synapses on hippocampal neurons. *Eur. J. Neurosci.* 16, 1313–1324.
- Li, L.-L., Ginet, V., Liu, X., Vergun, O., Tuittila, M., Mathieu, M., Bonny, C., Puyal, J.,

- Truttmann, A.C., Courtney, M.J., 2013. The nNOS-p38MAPK pathway is mediated by NOS1AP during neuronal death. *J. Neurosci. Off. J. Soc. Neurosci.* 33, 8185–8201. <https://doi.org/10.1523/JNEUROSCI.4578-12.2013>
- Li, N., Lee, B., Liu, R.-J., Banasr, M., Dwyer, J.M., Iwata, M., Li, X.-Y., Aghajanian, G., Duman, R.S., 2010. mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. *Science* 329, 959. <https://doi.org/10.1126/science.1190287>
- Li, N., Liu, R.-J., Dwyer, J.M., Banasr, M., Lee, B., Son, H., Li, X.-Y., Aghajanian, G., Duman, R.S., 2011. Glutamate NMDA receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol. Psychiatry* 69, 754–761. <https://doi.org/10.1016/j.biopsych.2010.12.015>
- Liao, Y., Huang, X., Wu, Q., Yang, C., Kuang, W., Du, M., Lui, S., Yue, Q., Chan, R.C., Kemp, G.J., others, 2013. Is depression a disconnection syndrome? Meta-analysis of diffusion tensor imaging studies in patients with MDD. *J. Psychiatry Neurosci. JPN* 38, 49.
- Licht, T., Eavri, R., Goshen, I., Shlomai, Y., Mizrahi, A., Keshet, E., 2010. VEGF is required for dendritogenesis of newly born olfactory bulb interneurons. *Development* 137, 261–271.
- Licznerski, P., Duman, R.S., 2013. Remodeling of axo-spinous synapses in the pathophysiology and treatment of depression. *Neuroscience* 251, 33–50.
- Liddel, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Münch, A.E., Chung, W.-S., Peterson, T.C., Wilton, D.K., Frouin, A., Napier, B.A., Panicker, N., Kumar, M., Buckwalter, M.S., Rowitch, D.H., Dawson, V.L., Dawson, T.M., Stevens, B., Barres, B.A., 2017. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature* 541, 481–487.
- Lima, A., Sardinha, V.M., Oliveira, A.F., Reis, M., Mota, C., Silva, M.A., Marques, F., Cerqueira, J.J., Pinto, L., Sousa, N., Oliveira, J.F., 2014. Astrocyte pathology in the prefrontal cortex impairs the cognitive function of rats. *Mol Psychiatry* 19, 834–841.
- Lin, L.-F.H., Doherty, D.H., Lile, J.D., Bektesh, S., Collins, F., 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Sci.-N. Y. THEN Wash.* 260, 1130–1130.
- Liston, C., McEwen, B.S., Casey, B.J., 2009. Psychosocial stress reversibly disrupts prefrontal processing and attentional control. *Proc. Natl. Acad. Sci.* 106, 912. <https://doi.org/10.1073/pnas.0807041106>
- Liston, C., Miller, M.M., Goldwater, D.S., Radley, J.J., Rocher, A.B., Hof, P.R., Morrison, J.H., McEwen, B.S., 2006. Stress-Induced Alterations in Prefrontal Cortical Dendritic Morphology Predict Selective Impairments in Perceptual Attentional Set-Shifting. *J. Neurosci.* 26, 7870. <https://doi.org/10.1523/JNEUROSCI.1184-06.2006>
- Liu, B., Teschemacher, A.G., Kasparov, S., 2017. Astroglia as a cellular target for neuroprotection and treatment of neuro-psychiatric disorders. *Glia*.
- Liu, F., Wu, J., Gong, Y., Wang, P., Zhu, L., Tong, L.-J., Chen, X., Ling, Y., Huang, C., 2017. Harmine produces antidepressant-like effects via restoration of astrocytic functions. *Prog. Neuropsychopharmacol. Biol. Psychiatry*.
- Liu, F., Wu, J., Gong, Y., Wang, P., Zhu, L., Tong, L.-J., Chen, X., Ling, Y., Huang, C., n.d. Harmine produces antidepressant-like effects via restoration of astrocytic functions. *Prog.*

Neuropsychopharmacol. Biol. Psychiatry. <https://doi.org/10.1016/j.pnpbp.2017.06.012>

Liu, H., Liu, G., Bi, Y., 2014. CNTF regulates neurite outgrowth and neuronal migration through JAK2/STAT3 and PI3K/Akt signaling pathways of DRG explants with gp120-induced neurotoxicity in vitro. *Neurosci. Lett.* 569, 110–115.

Liu, R.-J., Aghajanian, G.K., 2008. Stress blunts serotonin- and hypocretin-evoked EPSCs in prefrontal cortex: Role of corticosterone-mediated apical dendritic atrophy. *Proc. Natl. Acad. Sci. U. S. A.* 105, 359–364. <https://doi.org/10.1073/pnas.0706679105>

Liu, R.-J., Duman, C., Kato, T., Hare, B., Lopresto, D., Bang, E., Burgdorf, J., Moskal, J., Taylor, J., Aghajanian, G., Duman, R.S., 2017. GLYX-13 Produces Rapid Antidepressant Responses with Key Synaptic and Behavioral Effects Distinct from Ketamine. *Neuropsychopharmacology* 42, 1231–1242.

Liu, S.-G., Wang, Y.-M., Zhang, Y.-J., He, X.-J., Ma, T., Song, W., Zhang, Y.-M., n.d. ZL006 protects spinal cord neurons against ischemia-induced oxidative stress through AMPK-PGC-1 α -Sirt3 pathway. *Neurochem. Int.* <https://doi.org/10.1016/j.neuint.2017.04.005>

Longuemare, M.C., Hill, M.P., Swanson, R.A., 1994. Glycolysis can prevent non-synaptic excitatory amino acid release during hypoxia. *Neuroreport* 5, 1789–1792.

Louvar, H., Maccari, S., Vaiva, G., Darnaudéry, M., 2009. Prenatal stress exacerbates the impact of an aversive procedure on the corticosterone response to stress in female rats. *Psychoneuroendocrinology* 34, 786–790.

Lowery, L.A., Van Vactor, D., 2009. The trip of the tip: understanding the growth cone machinery. *Nat. Rev. Mol. Cell Biol.* 10, 332.

Lu, B., 2003. BDNF and activity-dependent synaptic modulation. *Learn. Mem.* 10, 86–98.

Lu, H., Zou, Q., Gu, H., Raichle, M.E., Stein, E.A., Yang, Y., 2012. Rat brains also have a default mode network. *Proc. Natl. Acad. Sci.* 109, 3979–3984.

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. *Neurol. Res.* 37, 803–808.

Luczynski, P., Moquin, L., Gratton, A., 2015. Chronic stress alters the dendritic morphology of callosal neurons and the acute glutamate stress response in the rat medial prefrontal cortex. *Stress* 18, 654–667. <https://doi.org/10.3109/10253890.2015.1073256>

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., Zhang, Y., Wu, H.-Y., Chang, L., Zhu, D.-Y., 2014. Interaction of nNOS with PSD-95 Negatively Controls Regenerative Repair after Stroke. *J. Neurosci.* 34, 13535. <https://doi.org/10.1523/JNEUROSCI.1305-14.2014>

Luo, L., 2002. Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. *Annu. Rev. Cell Dev. Biol.* 18, 601–635.

Lutgen, V., Narasipura, S.D., Sharma, A., Min, S., Al-Harthi, L., 2016. β -Catenin signaling positively regulates glutamate uptake and metabolism in astrocytes. *J. Neuroinflammation* 13, 242. <https://doi.org/10.1186/s12974-016-0691-7>

Luykx, J.J., Laban, K.G., Van Den Heuvel, M.P., Boks, M.P.M., Mandl, R.C.W., Kahn, R.S., Bakker, S.C., 2012. Region and state specific glutamate downregulation in major depressive

disorder: a meta-analysis of 1 H-MRS findings. *Neurosci. Biobehav. Rev.* 36, 198–205.

Ma, M., Ren, Q., Yang, C., Zhang, J., Yao, W., Dong, C., Ohgi, Y., Futamura, T., Hashimoto, K., 2016. Adjunctive treatment of brexpiprazole with fluoxetine shows a rapid antidepressant effect in social defeat stress model: Role of BDNF-TrkB signaling. *Sci. Rep.* 6, 39209. <https://doi.org/10.1038/srep39209>

MacDonald, J.F., Wojtowicz, J.M., 1982. The effects of L-glutamate and its analogues upon the membrane conductance of central murine neurones in culture. *Can. J. Physiol. Pharmacol.* 60, 282–296.

Machado-Vieira, R., Salvatore, G., DiazGranados, N., Zarate Jr., C.A., 2009. Ketamine and the next generation of antidepressants with a rapid onset of action. *Pharmacol. Ther.* 123, 143–150. <https://doi.org/10.1016/j.pharmthera.2009.02.010>

Madara, J.C., Levine, E.S., 2008. Presynaptic and postsynaptic NMDA receptors mediate distinct effects of brain-derived neurotrophic factor on synaptic transmission. *J. Neurophysiol.* 100, 3175–3184.

Madl, J.E., Burgesser, K., 1993. Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. *J. Neurosci.* 13, 4429–4444.

Maeng, S., Zarate, C.A., 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. *Curr. Psychiatry Rep.* 9, 467–474. <https://doi.org/10.1007/s11920-007-0063-1>

Maeng, S., Zarate Jr, C.A., Du, J., Schloesser, R.J., McCammon, J., Chen, G., Manji, H.K., 2008. Cellular Mechanisms Underlying the Antidepressant Effects of Ketamine: Role of α -Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid Receptors. *Stress Depress. Circuitry* 63, 349–352. <https://doi.org/10.1016/j.biopsych.2007.05.028>

Magariños, A.M., McEwen, B.S., Flügge, G., Fuchs, E., 1996. Chronic Psychosocial Stress Causes Apical Dendritic Atrophy of Hippocampal CA3 Pyramidal Neurons in Subordinate Tree Shrews. *J. Neurosci.* 16, 3534.

Magariños, A.M., Verdugo, J.M.G., McEwen, B.S., 1997. Chronic stress alters synaptic terminal structure in hippocampus. *Proc. Natl. Acad. Sci.* 94, 14002–14008.

Malinow, R., Malenka, R.C., 2002. AMPA receptor trafficking and synaptic plasticity. *Annu. Rev. Neurosci.* 25, 103–126.

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of Disease: astrocytes in neurodegenerative disease. *Nat Clin Pr. Neuro* 2, 679–689. <https://doi.org/10.1038/ncpneuro0355>

Martin, J.L., Brown, A.L., Balkowiec, A., 2012. Glia determine the course of brain-derived neurotrophic factor-mediated dendritogenesis and provide a soluble inhibitory cue to dendritic growth in the brainstem. *Neuroscience* 207, 333–346. <https://doi.org/10.1016/j.neuroscience.2012.01.013>

Martinez-Hernandez, A., Bell, K.P., Norenberg, M.D., 1977. Glutamine synthetase: glial localization in brain. *Science* 195, 1356–1358.

Marvin, J.S., Borghuis, B.G., Tian, L., Cichon, J., Harnett, M.T., Akerboom, J., Gordus, A.,

- Renninger, S.L., Chen, T.-W., Bargmann, C.I., Orger, M.B., Schreiter, E.R., Demb, J.B., Gan, W.-B., Hires, S.A., Looger, L.L., 2013. An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nat. Methods* 10, 162–170. <https://doi.org/10.1038/nmeth.2333>
- Massey, P.V., Bashir, Z.I., 2007. Long-term depression: multiple forms and implications for brain function. *Trends Neurosci.* 30, 176–184.
- Mathew, S.J., Murrough, J.W., aan het Rot, M., Collins, K.A., Reich, D.L., Charney, D.S., 2010. Riluzole for relapse prevention following intravenous ketamine in treatment-resistant depression: a pilot randomized, placebo-controlled continuation trial. *Int. J. Neuropsychopharmacol.* 13, 71–82.
- Mathews, D.C., Henter, I.D., Zarate, C.A., 2012. Targeting the Glutamatergic System to Treat Major Depressive Disorder: Rationale and Progress to Date. *Drugs* 72, 1313–1333. <https://doi.org/10.2165/11633130-000000000-00000>
- Matsutani, S., Yamamoto, N., 1998. GABAergic neuron-to-astrocyte signaling regulates dendritic branching in coculture. *Dev. Neurobiol.* 37, 251–264.
- Mattson, M.P., 2008. Glutamate and neurotrophic factors in neuronal plasticity and disease. *Ann. N. Y. Acad. Sci.* 1144, 97–112.
- Mattson, M.P., Dou, P., Kater, S.B., 1988. Outgrowth-regulating actions of glutamate in isolated hippocampal pyramidal neurons. *J. Neurosci.* 8, 2087–2100.
- Matus, A., Ackermann, M., Pehling, G., Byers, H.R., Fujiwara, K., 1982. High actin concentrations in brain dendritic spines and postsynaptic densities. *Proc. Natl. Acad. Sci.* 79, 7590–7594.
- Mauch, D.H., Nägler, K., Schumacher, S., Göritz, C., Müller, E.-C., Otto, A., Pfrieder, F.W., 2001. CNS synaptogenesis promoted by glia-derived cholesterol. *Science* 294, 1354–1357.
- Mayo, L., Trauger, S.A., Blain, M., Nadeau, M., Patel, B., Alvarez, J.I., Mascanfroni, I.D., Yeste, A., Kivisäkk, P., Kallas, K., 2014. Regulation of astrocyte activation by glycolipids drives chronic CNS inflammation. *Nat. Med.* 20, 1147–1156.
- McAllister, A.K., 2007. Dynamic aspects of CNS synapse formation. *Annu Rev Neurosci* 30, 425–450.
- McAllister, A.K., Katz, L.C., Lo, D.C., 1999. Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* 22, 295–318.
- McAllister, A.K., Lo, D.C., Katz, L.C., 1995. Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15, 791–803.
- McBean, G.J., 1994. Inhibition of the glutamate transporter and glial enzymes in rat striatum by the gliotoxin, alpha aminoadipate. *Br. J. Pharmacol.* 113, 536–540.
- McEwen, B.S., 2016. In pursuit of resilience: stress, epigenetics, and brain plasticity. *Ann. N. Y. Acad. Sci.* 1373, 56–64.
- McEwen, B.S., Morrison, J.H., 2013. The Brain on Stress: Vulnerability and Plasticity of the Prefrontal Cortex over the Life Course. *Neuron* 79, 16–29. <https://doi.org/10.1016/j.neuron.2013.06.028>
- McEwen, B.S., Nasca, C., Gray, J.D., 2016. Stress Effects on Neuronal Structure: Hippocampus, Amygdala, and Prefrontal Cortex. *Neuropsychopharmacology* 41, 3–23.

<https://doi.org/10.1038/npp.2015.171>

McIntosh, A.L., Gormley, S., Tozzi, L., Frodl, T., Harkin, A., 2017. Recent advances in translational magnetic resonance imaging in animal models of stress and depression. *Front. Cell. Neurosci.* 11.

McKeon, R.J., Schreiber, R.C., Rudge, J.S., 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. *J. Neurosci.* 11, 3398–3411.

McKinney, W.T., Bunney, W.E., 1969. Animal model of depression: I. Review of evidence: Implications for research. *Arch. Gen. Psychiatry* 21, 240–248.

McNally, L., Bhagwagar, Z., Hannestad, J., 2008. Inflammation, glutamate, and glia in depression: a literature review. *CNS Spectr.* 13, 501–510.

McNamee, E.N., Griffin, É.W., Ryan, K.M., Ryan, K.J., Heffernan, S., Harkin, A., Connor, T.J., 2010. Noradrenaline acting at β -adrenoceptors induces expression of IL-1 β and its negative regulators IL-1 α and IL-1RII, and drives an overall anti-inflammatory phenotype in rat cortex. *Neuropharmacology* 59, 37–48.

Medina, A., Watson, S.J., Bunney, W., Jr., Myers, R.M., Schatzberg, A., Barchas, J., Akil, H., Thompson, R.C., n.d. Evidence for alterations of the glial syncytial function in major depressive disorder. *J. Psychiatr. Res.* 72, 15–21. <https://doi.org/10.1016/j.jpsychires.2015.10.010>

Meissirel, C., de Almodovar, C.R., Knevels, E., Coulon, C., Chounlamountri, N., Segura, I., de Rossi, P., Vinckier, S., Anthonis, K., Deléglise, B., others, 2011. VEGF modulates NMDA receptors activity in cerebellar granule cells through Src-family kinases before synapse formation. *Proc. Natl. Acad. Sci.* 108, 13782–13787.

Ménard, C., Hodes, G.E., Russo, S.J., 2016. Pathogenesis of depression: Insights from human and rodent studies. *Neuroscience* 321, 138–162.

Mervaala, E., Föhr, J., Könönen, M., Valkonen-Korhonen, M., Vainio, P., Partanen, K., Partanen, J., Tiihonen, J., Viinamäki, H., Karjalainen, A.-K., others, 2000. Quantitative MRI of the hippocampus and amygdala in severe depression. *Psychol. Med.* 30, 117–125.

Merzenich, M.M., Van Vleet, T.M., Nahum, M., 2014. Brain plasticity-based therapeutics. *Front. Hum. Neurosci.* 8.

Metzger, F., Wiese, S., Sendtner, M., 1998. Effect of glutamate on dendritic growth in embryonic rat motoneurons. *J. Neurosci.* 18, 1735–1742.

Michael, N., Erfurth, A., Ohrmann, P., Arolt, V., Heindel, W., Pfleiderer, B., 2003. Neurotrophic Effects of Electroconvulsive Therapy: A Proton Magnetic Resonance Study of the Left Amygdalar Region in Patients with Treatment-Resistant Depression. *Neuropsychopharmacology* 28, 720–725.

Michelsen, K.A., van den Hove, D.L., Schmitz, C., Segers, O., Prickaerts, J., Steinbusch, H.W., 2007. Prenatal stress and subsequent exposure to chronic mild stress influence dendritic spine density and morphology in the rat medial prefrontal cortex. *BMC Neurosci.* 8, 107. <https://doi.org/10.1186/1471-2202-8-107>

Miguel-Hidalgo, J.J., Baucom, C., Dilley, G., Overholser, J.C., Meltzer, H.Y., Stockmeier, C.A., Rajkowska, G., 2000. Glial fibrillary acidic protein immunoreactivity in the prefrontal

cortex distinguishes younger from older adults in major depressive disorder. *Biol. Psychiatry* 48, 861–873. [https://doi.org/10.1016/S0006-3223\(00\)00999-9](https://doi.org/10.1016/S0006-3223(00)00999-9)

Miguel-Hidalgo, J.J., Waltzer, R., Whittom, A.A., Austin, M.C., Rajkowska, G., Stockmeier, C.A., 2010. Glial and glutamatergic markers in depression, alcoholism, and their comorbidity. *J. Affect. Disord.* 127, 230–240.

Miladinovic, T., Nashed, M.G., Singh, G., 2015. Overview of glutamatergic dysregulation in central pathologies. *Biomolecules* 5, 3112–3141.

Ming, G., Song, H., Berninger, B., Holt, C.E., Tessier-Lavigne, M., Poo, M., 1997. cAMP-dependent growth cone guidance by netrin-1. *Neuron* 19, 1225–1235.

Mitani, H., Shirayama, Y., Yamada, T., Maeda, K., Ashby, C.R., Kawahara, R., 2006. Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 30, 1155–1158. <https://doi.org/10.1016/j.pnpbp.2006.03.036>

Miyamoto, E., 2006. Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *J. Pharmacol. Sci.* 100, 433–442.

Moench, K.M., Maroun, M., Kavushansky, A., Wellman, C., 2016. Alterations in neuronal morphology in infralimbic cortex predict resistance to fear extinction following acute stress. *Neurobiol. Stress* 3, 23–33. <https://doi.org/10.1016/j.ynstr.2015.12.002>

Moghaddam, B., 1993. Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. *J. Neurochem.* 60, 1650–1657.

Moghaddam, B., Adams, B., Verma, A., Daly, D., 1997. Activation of Glutamatergic Neurotransmission by Ketamine: A Novel Step in the Pathway from NMDA Receptor Blockade to Dopaminergic and Cognitive Disruptions Associated with the Prefrontal Cortex. *J. Neurosci.* 17, 2921.

Molofsky, A.V., Krennick, R., Ullian, E., Tsai, H., Deneen, B., Richardson, W.D., Barres, B.A., Rowitch, D.H., 2012. Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev.* 26, 891–907. <https://doi.org/10.1101/gad.188326.112>

Moncada, S., Bolaños, J.P., 2006. Nitric oxide, cell bioenergetics and neurodegeneration. *J. Neurochem.* 97, 1676–1689.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* 43, 109.

Monnerie, H., Shashidhara, S., Le Roux, P.D., 2003. Effect of excess extracellular glutamate on dendrite growth from cerebral cortical neurons at 3 days in vitro: Involvement of NMDA receptors. *J. Neurosci. Res.* 74, 688–700.

Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B., Seeburg, P.H., 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12, 529–540.

Morales Rico, A., López Mendoza, A., Bravo Durán, D.A., de la Luz Torres, H., Arias Mendoza, G., Silva Gómez, A.B., 2015. The effects of chronic restraint on the morphology of ventral CA1 neurons in female Long Evans rats. *Stress* 18, 67–75. <https://doi.org/10.3109/10253890.2014.974029>

- Moser, E., Stadlbauer, A., Windischberger, C., Quick, H.H., Ladd, M.E., 2009. Magnetic resonance imaging methodology. *Eur. J. Nucl. Med. Mol. Imaging* 36, 30.
- Muhammad, A., Carroll, C., Kolb, B., 2012. Stress during development alters dendritic morphology in the nucleus accumbens and prefrontal cortex. *Neuroscience* 216, 103–109. <https://doi.org/10.1016/j.neuroscience.2012.04.041>
- Muhammad, A., Kolb, B., 2011. Maternal separation altered behavior and neuronal spine density without influencing amphetamine sensitization. *Behav. Brain Res.* 223, 7–16. <https://doi.org/10.1016/j.bbr.2011.04.015>
- Muir, K.W., 2006. Glutamate-based therapeutic approaches: clinical trials with NMDA antagonists. *Neurosciences* 6, 53–60. <https://doi.org/10.1016/j.coph.2005.12.002>
- Muñoz, F.J., Godoy, J.A., Cerpa, W., Poblete, I.M., Huidobro-Toro, J.P., Inestrosa, N.C., 2014. Wnt-5a increases NO and modulates NMDA receptor in rat hippocampal neurons. *Biochem. Biophys. Res. Commun.* 444, 189–194.
- Murgatroyd, P.N., 1970. Theory of space-charge-limited current enhanced by Frenkel effect. *J. Phys. Appl. Phys.* 3, 151.
- Murmu, M.S., Salomon, S., Biala, Y., Weinstock, M., Braun, K., Bock, J., 2006. Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. *Eur J Neurosci* 24. <https://doi.org/10.1111/j.1460-9568.2006.05024.x>
- Musazzi, L., Milanese, M., Farisello, P., Zappettini, S., Tardito, D., Barbiero, V.S., Bonifacino, T., Mallei, A., Baldelli, P., Racagni, G., others, 2010. Acute stress increases depolarization-evoked glutamate release in the rat prefrontal/frontal cortex: the dampening action of antidepressants. *PloS One* 5, e8566.
- Mychasiuk, R., Zahir, S., Schmold, N., Ilnytsky, S., Kovalchuk, O., Gibb, R., 2012. Parental enrichment and offspring development: modifications to brain, behavior and the epigenome. *Behav. Brain Res.* 228, 294–298.
- Nagahara, A.H., Merrill, D.A., Coppola, G., Tsukada, S., Schroeder, B.E., Shaked, G.M., Wang, L., Blesch, A., Kim, A., Conner, J.M., 2009. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat. Med.* 15, 331–337.
- Nägler, K., Mauch, D.H., Pfriger, F.W., 2001. Glia-derived signals induce synapse formation in neurones of the rat central nervous system. *J. Physiol.* 533, 665–679.
- Nedergaard, M., Ransom, B., Goldman, S.A., 2003. New roles for astrocytes: Redefining the functional architecture of the brain. *Trends Neurosci.* 26, 523–530. <https://doi.org/10.1016/j.tins.2003.08.008>
- Nestler, E.J., Barrot, M., DiLeone, R.J., Eisch, A.J., Gold, S.J., Monteggia, L.M., 2002a. Neurobiology of Depression. *Neuron* 34, 13–25. [https://doi.org/10.1016/S0896-6273\(02\)00653-0](https://doi.org/10.1016/S0896-6273(02)00653-0)
- Nestler, E.J., Gould, E., Manji, H., 2002b. Preclinical models: status of basic research in depression. *Biol. Psychiatry* 52, 503–528.
- Niciu, M.J., Kelmendi, B., Sanacora, G., 2012. Overview of Glutamatergic Neurotransmission in the Nervous System. *Pharmacol. Biochem. Behav.* 100, 656–664.

<https://doi.org/10.1016/j.pbb.2011.08.008>

Nimchinsky, E.A., Sabatini, B.L., Svoboda, K., 2002. Structure and function of dendritic spines. *Annu. Rev. Physiol.* 64, 313–353.

Nishimura, R.N., Santos, D., Fu, S.-T., Dwyer, B.E., 200. Induction of cell death by L-alpha-amino adipic acid exposure in cultured rat astrocytes: relationship to protein synthesis. *Neurotoxicology* 21, 313–320.

Nott, A., Watson, P.M., Robinson, J.D., Crepaldi, L., Riccio, A., 2008. S-Nitrosylation of histone deacetylase 2 induces chromatin remodelling in neurons. *Nature* 455, 411.

O'Dell, T.J., Hawkins, R.D., Kandel, E.R., Arancio, O., 1991. Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger. *Proc. Natl. Acad. Sci.* 88, 11285–11289.

Oh, W.C., Hill, T.C., Zito, K., 2013. Synapse-specific and size-dependent mechanisms of spine structural plasticity accompanying synaptic weakening. *Proc. Natl. Acad. Sci.* 110, E305–E312.

Ohsawa, K., Imai, Y., Sasaki, Y., Kohsaka, S., 2004. Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. *J. Neurochem.* 88, 844–856.

Okabe, S., Miwa, A., Okado, H., 2001. Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J. Neurosci.* 21, 6105–6114.

O'Kusky, J.R., Ye, P., D'Ercole, A.J., 2000. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J. Neurosci.* 20, 8435–8442.

Olney, J.W., de Gubareff, T., Collins, J.F., 1980. Stereospecificity of the gliotoxic and anti-neurotoxic actions of alpha-amino adipate. *Neurosci. Lett.* 19, 277–282. [https://doi.org/10.1016/0304-3940\(80\)90273-6](https://doi.org/10.1016/0304-3940(80)90273-6)

O'Neill, E., Kwok, B., Day, J.S., Connor, T.J., Harkin, A., 2016. Amitriptyline protects against TNF- α -induced atrophy and reduction in synaptic markers via a Trk-dependent mechanism. *Pharmacol. Res. Perspect.* 4.

Opler, L.A., Medalia, A., Opler, M.G., Stahl, S.M., 2014. Pharmacotherapy of cognitive deficits in schizophrenia. *CNS Spectr.* 19, 142–156.

Orthmann-Murphy, J.L., Abrams, C.K., Scherer, S.S., 2008. Gap Junctions Couple Astrocytes and Oligodendrocytes. *J. Mol. Neurosci.* MN 35, 101–116. <https://doi.org/10.1007/s12031-007-9027-5>

Ota, K.T., Duman, R.S., 2013. Environmental and pharmacological modulation of cellular plasticity: role in the pathophysiology and treatment of depression. *Neurobiol. Dis.* 0, 28–37. <https://doi.org/10.1016/j.nbd.2012.05.022>

Otal, R., Martínez, A., Soriano, E., 2005. Lack of TrkB and TrkC signaling alters the synaptogenesis and maturation of mossy fiber terminals in the hippocampus. *Cell Tissue Res.* 319, 349–358.

Ouyang, Y.-B., Voloboueva, L.A., Xu, L.-J., Giffard, R.G., 2007. Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *J. Neurosci. Off. J. Soc. Neurosci.* 27, 4253–4260.

<https://doi.org/10.1523/JNEUROSCI.0211-07.2007>

Padival, M.A., Blume, S.R., Rosenkranz, J.A., 2013. Repeated restraint stress exerts different impact on structure of neurons in the lateral and basal nuclei of the amygdala. *Neuroscience* 246, 230–242. <https://doi.org/10.1016/j.neuroscience.2013.04.061>

Padival, M.A., Blume, S.R., Vantrease, J.E., Rosenkranz, J.A., 2015. Qualitatively different effect of repeated stress during adolescence on principal neuron morphology across lateral and basal nuclei of the rat amygdala. *Neuroscience* 291, 128–145. <https://doi.org/10.1016/j.neuroscience.2015.02.012>

Palop, J.J., Mucke, L., 2010. Synaptic depression and aberrant excitatory network activity in Alzheimer's disease: two faces of the same coin? *Neuromolecular Med.* 12, 48–55.

Panickar, K.S., Norenberg, M.D., 2005. Astrocytes in cerebral ischemic injury: Morphological and general considerations. *Glia* 50, 287–298. <https://doi.org/10.1002/glia.20181>

Papp, M., Moryl, E., Willner, P., 1996. Pharmacological validation of the chronic mild stress model of depression. *Eur. J. Pharmacol.* 296, 129–136.

Pardo, C.A., Eberhart, C.G., 2007. The neurobiology of autism. *Brain Pathol.* 17, 434–447.

Park, H., Poo, M., 2013. Neurotrophin regulation of neural circuit development and function. *Nat. Rev. Neurosci.* 14, 7.

Parnass, Z., Tashiro, A., Yuste, R., 2000. Analysis of spine morphological plasticity in developing hippocampal pyramidal neurons. *Hippocampus* 10, 561–568. [https://doi.org/10.1002/1098-1063\(2000\)10:5<561::AID-HIPO6>3.0.CO;2-X](https://doi.org/10.1002/1098-1063(2000)10:5<561::AID-HIPO6>3.0.CO;2-X)

Parpura, V., Heneka, M.T., Montana, V., Oliet, S.H., Schousboe, A., Haydon, P.G., Stout, R.F., Spray, D.C., Reichenbach, A., Pannicke, T., others, 2012. Glial cells in (patho) physiology. *J. Neurochem.* 121, 4–27.

Parrish, J.Z., Emoto, K., Kim, M.D., Jan, Y.N., 2007. Mechanisms that regulate establishment, maintenance, and remodeling of dendritic fields. *Annu Rev Neurosci* 30, 399–423.

Patel, M.N., McNamara, J.O., 1995. Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. *Neuroscience* 69, 763–770.

Pav, M., Kovaru, H., Fiserova, A., Havrdova, E., Lisa, V., 2008. Neurobiological aspects of depressive disorder and antidepressant treatment: role of glia. *Physiol. Res.* 57, 151.

Pawlak, R., Rao, B.S.S., Melchor, J.P., Chattarji, S., McEwen, B., Strickland, S., 2005. Tissue plasminogen activator and plasminogen mediate stress-induced decline of neuronal and cognitive functions in the mouse hippocampus. *Proc. Natl. Acad. Sci. U. S. A.* 102, 18201–18206. <https://doi.org/10.1073/pnas.0509232102>

Pehrson, A.L., Sanchez, C., 2015. Altered γ -aminobutyric acid neurotransmission in major depressive disorder: a critical review of the supporting evidence and the influence of serotonergic antidepressants. *Drug Des. Devel. Ther.* 9, 603.

Pekny, M., Wilhelmsson, U., Bogestål, Y.R., Pekna, M., 2007. The role of astrocytes and complement system in neural plasticity. *Int. Rev. Neurobiol.* 82, 95–111.

Pekny, M., Wilhelmsson, U., Pekna, M., 2014. The dual role of astrocyte activation and

reactive gliosis. *Neurosci. Lett.*, Astrocytes in the Injured Brain: Is Gliosis Adaptive or Maladaptive? 565, 30–38. <https://doi.org/10.1016/j.neulet.2013.12.071>

Penazzi, L., Bakota, L., Brandt, R., 2016. Microtubule Dynamics in Neuronal Development, Plasticity, and Neurodegeneration. *Int. Rev. Cell Mol. Biol.* 321, 89–169. <https://doi.org/10.1016/bs.ircmb.2015.09.004>

Pennybaker, S.J., Niciu, M.J., Luckenbaugh, D.A., Zarate, C.A., 2017. Symptomatology and predictors of antidepressant efficacy in extended responders to a single ketamine infusion. *J. Affect. Disord.* 208, 560–566. <https://doi.org/10.1016/j.jad.2016.10.026>

Pereira Diniz, L., Tortelli, V., Matias, I., Morgado, J., Bérnago Araujo, A.P., Melo, H.M., Seixas da Silva, G.S., Alves-Leon, S.V., de Souza, J.M., Ferreira, S.T., De Felice, F.G., Gomes, F.C.A., 2017. Astrocyte Transforming Growth Factor Beta 1 Protects Synapses against A β Oligomers in Alzheimer's Disease Model. *J. Neurosci.* 37, 6797–6809.

Pessoa, L., 2008. On the relationship between emotion and cognition. *Nat Rev Neurosci* 9, 148–158. <https://doi.org/10.1038/nrn2317>

Pfriege, F.W., 2003. Role of cholesterol in synapse formation and function. *Cholest.-Rich Domains* 1610, 271–280. [https://doi.org/10.1016/S0005-2736\(03\)00024-5](https://doi.org/10.1016/S0005-2736(03)00024-5)

Pfriege, F.W., Barres, B.A., 1997. Synaptic Efficacy Enhanced by Glial Cells in Vitro. *Science* 277, 1684–1687.

Phillips, M., Pozzo-Miller, L., 2015. Dendritic spine dysgenesis in autism related disorders. *Neurosci. Lett.* 601, 30–40.

Podestá, M.F., Yam, P., Codagnone, M.G., Uccelli, N.A., Colman, D., Reínés, 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.

Polleux, F., Snider, W., 2010. Initiating and growing an axon. *Cold Spring Harb. Perspect. Biol.* 2, a001925.

Popoli, M., Yan, Z., McEwen, B.S., Sanacora, G., 2012. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat. Rev. Neurosci.* 13, 22–37.

PORSOLT, R.D., LE PICHON, M., JALFRE, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266, 730–732. <https://doi.org/10.1038/266730a0>

Powell, S.K., Rivas, R.J., Rodriguez-Boulan, E., Hatten, M.E., 1997. Development of polarity in cerebellar granule neurons. *J. Neurobiol.* 32, 223–236.

Pozzo-Miller, L.D., Gottschalk, W., Zhang, L., McDermott, K., Du, J., Gopalakrishnan, R., Oho, C., Sheng, Z.-H., Lu, B., 1999. Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J. Neurosci.* 19, 4972–4983.

Previtera, M.L., Langhammer, C.G., Langrana, N.A., Firestein, B.L., 2010. Regulation of dendrite arborization by substrate stiffness is mediated by glutamate receptors. *Ann. Biomed. Eng.* 38, 3733–3743.

- Qiao, H., An, S.-C., Ren, W., Ma, X.-M., 2014. Progressive alterations of hippocampal CA3-CA1 synapses in an animal model of depression. *Behav. Brain Res.* 275, 191–200. <https://doi.org/10.1016/j.bbr.2014.08.040>
- Radley, Jason J, Anderson, R.M., Hamilton, B.A., Alcock, J.A., Romig-Martin, S.A., 2013. Chronic Stress-Induced Alterations of Dendritic Spine Subtypes Predict Functional Decrements in an Hypothalamo–Pituitary–Adrenal-Inhibitory Prefrontal Circuit. *J. Neurosci.* 33, 14379–14391. <https://doi.org/10.1523/JNEUROSCI.0287-13.2013>
- Radley, J.J., Arias, C.M., Sawchenko, P.E., 2006. Regional differentiation of the medial prefrontal cortex in regulating adaptive responses to acute emotional stress. *J Neurosci* 26. <https://doi.org/10.1523/JNEUROSCI.4297-06.2006>
- Radley, Jason J., Rocher, A.B., Rodriguez, A., Ehlenberger, D.B., Dammann, M., McEwen, B.S., Morrison, J.H., Wearne, S.L., Hof, P.R., 2008. Repeated stress alters dendritic spine morphology in the rat medial prefrontal cortex. *J. Comp. Neurol.* 507, 1141–1150. <https://doi.org/10.1002/cne.21588>
- Rajkowska, G, Miguel-Hidalgo, J., 2007. Gliogenesis and Glial Pathology in Depression. *CNS Neurol. Disord. Drug Targets* 6, 219–233.
- Rajkowska, G., Miguel-Hidalgo, J.J., Wei, J., Dilley, G., Pittman, S.D., Meltzer, H.Y., Overholser, J.C., Roth, B.L., Stockmeier, C.A., 1999. Morphometric evidence for neuronal and glial prefrontal cell pathology in major depression. *Biol. Psychiatry* 45, 1085–1098.
- Rajkowska, G., Stockmeier, C.A., 2013. Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. *Curr. Drug Targets* 14, 1225–1236.
- Ramos-Fernández, E., Tajés, M., Gerard, I.-R., Vargas, L., Busquets-García, A., Bosch-Morató, M., Guivernau, B., Valls-Comamala, V., Gomis, M., Grau, C., others, 2016. Glutamatergic stimulation induces GluN2B translation by the nitric oxide-Heme-Regulated eIF2 α kinase in cortical neurons. *Oncotarget* 7, 58876.
- Ranjan, A., Mallick, B.N., 2010. A modified method for consistent and reliable Golgi–Cox staining in significantly reduced time. *Front. Neurol.* 1.
- Ranscht, B., 2000. Cadherins: molecular codes for axon guidance and synapse formation. *Int. J. Dev. Neurosci.* 18, 643–651.
- Rao, M.S., Shetty, A.K., 2004. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *Eur. J. Neurosci.* 19, 234–246.
- Rash, J.E., Yasumura, T., Dudek, F.E., Nagy, J.I., 2001. Cell-Specific Expression of Connexins and Evidence of Restricted Gap Junctional Coupling between Glial Cells and between Neurons. *J. Neurosci. Off. J. Soc. Neurosci.* 21, 1983–2000.
- Raymond, G.V., Bauman, M.L., Kemper, T.L., 1995. Hippocampus in autism: a Golgi analysis. *Acta Neuropathol. (Berl.)* 91, 117–119.
- Read, D.E., Gorman, A.M., 2009. Involvement of Akt in neurite outgrowth. *Cell. Mol. Life Sci.* 66, 2975–2984.
- Redmond, L., Kashani, A.H., Ghosh, A., 2002. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron* 34, 999–1010.

- Riccio, A., Alvania, R.S., Lonze, B.E., Ramanan, N., Kim, T., Huang, Y., Dawson, T.M., Snyder, S.H., Ginty, D.D., 2006. A nitric oxide signaling pathway controls CREB-mediated gene expression in neurons. *Mol. Cell* 21, 283–294.
- Rigato, F., Garwood, J., Calco, V., Heck, N., Faivre-Sarrailh, C., Faissner, A., 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. *J. Neurosci.* 22, 6596–6609.
- Ripley, B., Otto, S., Tiglio, K., Williams, M.E., Ghosh, A., 2011. Regulation of synaptic stability by AMPA receptor reverse signaling. *Proc. Natl. Acad. Sci.* 108, 367–372.
- Robson, M.J., Elliott, M., Seminerio, M.J., Matsumoto, R.R., 2012a. Evaluation of sigma (σ) receptors in the antidepressant-like effects of ketamine in vitro and in vivo. *Eur. Neuropsychopharmacol.* 22, 308–317.
- Robson, M.J., Elliott, M., Seminerio, M.J., Matsumoto, R.R., 2012b. Evaluation of sigma (σ) receptors in the antidepressant-like effects of ketamine in vitro and in vivo. *Eur. Neuropsychopharmacol.* 22, 308–317.
- Rosenberg, D.R., MacMaster, F.P., Mirza, Y., Smith, J.M., Easter, P.C., Banerjee, S.P., Bhandari, R., Boyd, C., Lynch, M., Rose, M., Ivey, J., Villafuerte, R.A., Moore, G.J., Renshaw, P., 2005. Reduced Anterior Cingulate Glutamate in Pediatric Major Depression: A Magnetic Resonance Spectroscopy Study. *Biol. Psychiatry* 58, 700–704. <https://doi.org/10.1016/j.biopsych.2005.05.007>
- Rosoklija, G., Toomayan, G., Ellis, S.P., Keilp, J., Mann, J.J., Latov, N., Hays, A.P., Dwork, A.J., 2000. Structural abnormalities of subicular dendrites in subjects with schizophrenia and mood disorders: preliminary findings. *Arch. Gen. Psychiatry* 57, 349–356.
- Rothman, D.L., De Feyter, H.M., Maciejewski, P.K., Behar, K.L., 2012. Is there in vivo evidence for amino acid shuttles carrying ammonia from neurons to astrocytes? *Neurochem. Res.* 37, 2597–2612. <https://doi.org/10.1007/s11064-012-0898-7>
- Sacher, J., Neumann, J., Fünfstück, T., Soliman, A., Villringer, A., Schroeter, M.L., 2012. Mapping the depressed brain: a meta-analysis of structural and functional alterations in major depressive disorder. *J. Affect. Disord.* 140, 142–148.
- Sakharkar, A.J., Vetreno, R.P., Zhang, H., Kokare, D.M., Crews, F.T., Pandey, S.C., 2016. A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. *Brain Struct. Funct.* 221, 4691–4703. <https://doi.org/10.1007/s00429-016-1196-y>
- Salama-Cohen, P., Arévalo, M.-Á., Grantyn, R., Rodríguez-Tébar, A., 2006. Notch and NGF/p75NTR control dendrite morphology and the balance of excitatory/inhibitory synaptic input to hippocampal neurones through Neurogenin 3. *J. Neurochem.* 97, 1269–1278.
- Salinas, P.C., 2012. Wnt signaling in the vertebrate central nervous system: from axon guidance to synaptic function. *Cold Spring Harb. Perspect. Biol.* 4, a008003.
- Samdani, A.F., Newcamp, C., Resink, A., Facchinetti, F., Hoffman, B.E., Dawson, V.L., Dawson, T.M., 1997. Differential susceptibility to neurotoxicity mediated by neurotrophins and neuronal nitric oxide synthase. *J. Neurosci.* 17, 4633–4641.
- Sanacora, G., Gueorguieva, R., Epperson, C.N., Wu, Y.-T., Appel, M., Rothman, D.L., Krystal, J.H., Mason, G.F., 2004. Subtype-specific alterations of γ -aminobutyric acid and

glutamate in patients with major depression. *Arch. Gen. Psychiatry* 61, 705–713.

Sanacora, G., Treccani, G., Popoli, M., 2012. Towards a glutamate hypothesis of depression: An emerging frontier of neuropsychopharmacology for mood disorders. *Anxiety Depress.* 62, 63–77. <https://doi.org/10.1016/j.neuropharm.2011.07.036>

Sanacora, G., Zarate, C.A., Krystal, J.H., Manji, H.K., 2008. Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nat Rev Drug Discov* 7, 426–437. <https://doi.org/10.1038/nrd2462>

Sanchez, A.L., Matthews, B.J., Meynard, M.M., Hu, B., Javed, S., Cohen-Cory, S., 2006. BDNF increases synapse density in dendrites of developing tectal neurons in vivo. *Development* 133, 2477–2486.

Sarder, M., Abe, K., Saito, H., Nishiyama, N., 1996. Comparative effect of IL-2 and IL-6 on morphology of cultured hippocampal neurons from fetal rat brain. *Brain Res.* 715, 9–16.

Sato, K., 2013. Disruption of spine homeostasis causes depression. *Med. Hypotheses* 81, 5–9. <https://doi.org/10.1016/j.mehy.2013.03.025>

Sattler, R., Xiong, Z., Lu, W.-Y., Hafner, M., MacDonald, J.F., Tymianski, M., 1999. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. *Science* 284, 1845–1848.

Schechter, L.E., Ring, R.H., Beyer, C.E., Hughes, Z.A., Khawaja, X., Malberg, J.E., Rosenzweig-Lipson, S., 2005. Innovative approaches for the development of antidepressant drugs: current and future strategies. *NeuroRx* 2, 590–611.

Schildkraut, J.J., 1965. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am. J. Psychiatry* 122, 509–522. <https://doi.org/10.1176/ajp.122.5.509>

Schmaal, L., Veltman, D.J., van Erp, T.G., Sämann, P.G., Frodl, T., Jahanshad, N., Loehrer, E., Tiemeier, H., Hofman, A., Niessen, W.J., others, 2016. Subcortical brain alterations in major depressive disorder: findings from the ENIGMA Major Depressive Disorder working group. *Mol. Psychiatry* 21, 806.

Scholz, J., Woolf, C.J., 2007. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 10, 1361–1368. <https://doi.org/10.1038/nn1992>

Schroeter, M.L., Abdul-Khaliq, H., Krebs, M., Diefenbacher, A., Blasig, I.E., 2008. Serum markers support disease-specific glial pathology in major depression. *J. Affect. Disord.* 111, 271–280. <https://doi.org/10.1016/j.jad.2008.03.005>

Schroeter, M.L., Sacher, J., Steiner, J., Schoenknecht, P., Mueller, K., 2013. Serum S100B Represents a New Biomarker for Mood Disorders. *Curr. Drug Targets* 14, 1237–1248. <https://doi.org/10.2174/13894501113149990014>

Schuman, E.M., Madison, D.V., 1991. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254, 1503–1506.

Schumann, C.M., Hamstra, J., Goodlin-Jones, B.L., Lotspeich, L.J., Kwon, H., Buonocore, M.H., Lammers, C.R., Reiss, A.L., Amaral, D.G., 2004. The amygdala is enlarged in children but not adolescents with autism; the hippocampus is enlarged at all ages. *J. Neurosci.* 24, 6392–6401.

Schwamborn, J.C., Püschel, A.W., 2004. The sequential activity of the GTPases Rap1B and

- Cdc42 determines neuronal polarity. *Nat. Neurosci.* 7, 923.
- Scott, E.K., Luo, L., 2001. How do dendrites take their shape? *Nat. Neurosci.* 4, 359.
- Sebastian, V., Estil, J.B., Chen, D., Schrott, L.M., Serrano, P.A., 2013. Acute Physiological Stress Promotes Clustering of Synaptic Markers and Alters Spine Morphology in the Hippocampus. *PLoS ONE* 8. <https://doi.org/10.1371/journal.pone.0079077>
- Segmiller, F., R  ther, T., Linhardt, A., Padberg, F., Berger, M., Pogarell, O., M  ller, H.-J., Kohler, C., Sch  le, C., 2013. Repeated S-ketamine Infusions in Therapy Resistant Depression: A Case Series. *J. Clin. Pharmacol.* 53, 996–998.
- Seifert, G., Schilling, K., Steinh  user, C., 2006. Astrocyte dysfunction in neurological disorders: a molecular perspective. *Nat. Rev. Neurosci.* 7, 194–206. <https://doi.org/10.1038/nrn1870>
- Selvakumar, B., Jenkins, M.A., Hussain, N.K., Huganir, R.L., Traynelis, S.F., Snyder, S.H., 2013. S-nitrosylation of AMPA receptor GluA1 regulates phosphorylation, single-channel conductance, and endocytosis. *Proc. Natl. Acad. Sci.* 110, 1077–1082.
- Serulle, Y., Zhang, S., Ninan, I., Puzzo, D., McCarthy, M., Khatri, L., Arancio, O., Ziff, E.B., 2007. A GluR1-cGKII interaction regulates AMPA receptor trafficking. *Neuron* 56, 670–688.
- Sexton, C.E., Mackay, C.E., Ebmeier, K.P., 2009. A systematic review of diffusion tensor imaging studies in affective disorders. *Biol. Psychiatry* 66, 814–823.
- Shankar, G.M., Bloodgood, B.L., Townsend, M., Walsh, D.M., Selkoe, D.J., Sabatini, B.L., 2007. Natural oligomers of the Alzheimer amyloid- β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J. Neurosci.* 27, 2866–2875.
- Sheline, Y.I., Wang, P.W., Gado, M.H., Csernansky, J.G., Vannier, M.W., 1996. Hippocampal atrophy in recurrent major depression. *Proc. Natl. Acad. Sci.* 93, 3908–3913.
- Shen, K., Cowan, C.W., 2010. Guidance molecules in synapse formation and plasticity. *Cold Spring Harb. Perspect. Biol.* 2, a001842.
- Shen, W., Wu, B., Zhang, Z., Dou, Y., Rao, Z., Chen, Y., Duan, S., 2006. Activity-induced rapid synaptic maturation mediated by presynaptic cdc42 signaling. *Neuron* 50, 401–414.
- Shepherd, J.D., Huganir, R.L., 2007. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol* 23, 613–643.
- Sherwin, E., Gigliucci, V., Harkin, A., 2017a. Regional specific modulation of neuronal activation associated with nitric oxide synthase inhibitors in an animal model of antidepressant activity. *Behav. Brain Res.* 316, 18–28. <https://doi.org/10.1016/j.bbr.2016.08.049>
- Sherwin, E., Lennon, A., Harkin, A., 2017b. Regional specific modulation of stress-induced neuronal activation associated with the PSD95/NOS interaction inhibitor ZL006 in the Wistar Kyoto rat. *Int. J. Neuropsychopharmacol.*
- Shu, Y., Xu, T., 2017. Chronic Social Defeat Stress Modulates Dendritic Spines Structural Plasticity in Adult Mouse Frontal Association Cortex. *Neural Plast.* 2017, 6207873. <https://doi.org/10.1155/2017/6207873>
- Siero, J.C., Hermes, D., Hoogduin, H., Luijten, P.R., Ramsey, N.F., Petridou, N., 2014.

BOLD matches neuronal activity at the mm scale: a combined 7T fMRI and ECoG study in human sensorimotor cortex. *Neuroimage* 101, 177–184.

Silver, J., Miller, J.H., 2004. Regeneration beyond the glial scar. *Nat. Rev. Neurosci.* 5, 146.

Simard, M., Nedergaard, M., 2004. The neurobiology of glia in the context of water and ion homeostasis. *Brain Water Homeost.* 129, 877–896.
<https://doi.org/10.1016/j.neuroscience.2004.09.053>

Simon, B.M., Malisan, F., Testi, R., Nicotera, P., Leist, M., 2002. Disialoganglioside GD3 is released by microglia and induces oligodendrocyte apoptosis. *Cell Death Differ.* 9, 758.

Smith, A.E., Xu, Z., Lai, Y.Y., Kulkarni, P.M., Thakur, G.A., Hohmann, A.G., Crystal, J.D., 2016. Source memory in rats is impaired by an NMDA receptor antagonist but not by PSD95-nNOS protein-protein interaction inhibitors. *Behav. Brain Res.* 305, 23–29.
<https://doi.org/10.1016/j.bbr.2016.02.021>

Smith-Thomas, L.C., Fok-Seang, J., Stevens, J., Du, J.-S., Muir, E., Faissner, A., Geller, H.M., Rogers, J.H., Fawcett, J.W., 1994. An inhibitor of neurite outgrowth produced by astrocytes. *J. Cell Sci.* 107, 1687–1695.

Snow, D.M., Lemmon, V., Carrino, D.A., Caplan, A.I., Silver, J., 1990. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Exp. Neurol.* 109, 111–130.

Soetanto, A., Wilson, R.S., Talbot, K., Un, A., Schneider, J.A., Sobiesk, M., Kelly, J., Leurgans, S., Bennett, D.A., Arnold, S.E., 2010. Association of anxiety and depression with microtubule-associated protein 2-and synaptopodin-immunolabeled dendrite and spine densities in hippocampal CA3 of older humans. *Arch. Gen. Psychiatry* 67, 448–457.

Sofroniew, M.V., 2009. Molecular dissection of reactive astrogliosis and glial scar formation. *Trends Neurosci.* 32, 638–647.

Sofroniew, M.V., Vinters, H.V., 2010. Astrocytes: biology and pathology. *Acta Neuropathol. (Berl.)* 119, 7–35.

Lee, S., Kim, G., Kim, S., 2013. Glutamine deficiency in the prefrontal cortex increases depressive-like behaviours in male mice. *J. Psychiatry Neurosci. JPN* 38, 183.

Song, H., Poo, M., 1999. Signal transduction underlying growth cone guidance by diffusible factors. *Curr. Opin. Neurobiol.* 9, 355–363.

Song, J.-Y., Ichtchenko, K., Südhof, T.C., Brose, N., 1999. Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc. Natl. Acad. Sci. U. S. A.* 96, 1100–1105.

Sousa, N., Lukoyanov, N.V., Madeira, M.D., Almeida, O.F.X., Paula-Barbosa, M.M., 2000. Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience* 97, 253–266.
[https://doi.org/10.1016/S0306-4522\(00\)00050-6](https://doi.org/10.1016/S0306-4522(00)00050-6)

Stankiewicz, T.R., Linseman, D.A., 2014. Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration. *Front. Cell. Neurosci.* 8, 314.
<https://doi.org/10.3389/fncel.2014.00314>

Steele, J.D., Currie, J., Lawrie, S.M., Reid, I., 2007. Prefrontal cortical functional abnormality in major depressive disorder: a stereotactic meta-analysis. *J. Affect. Disord.* 101,

1–11.

Steinert, J.R., Chernova, T., Forsythe, I.D., 2010. Nitric oxide signaling in brain function, dysfunction, and dementia. *The Neuroscientist* 16, 435–452.

Stephan, K.E., Baldeweg, T., Friston, K.J., 2006. Synaptic plasticity and dysconnection in schizophrenia. *Biol. Psychiatry* 59, 929–939.

Stephan, K.E., Friston, K.J., Frith, C.D., 2009. Dysconnection in schizophrenia: from abnormal synaptic plasticity to failures of self-monitoring. *Schizophr. Bull.* 35, 509–527.

Steru, L., Chermat, R., Thierry, B., Simon, P., 1985. The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl.)* 85, 367–370.

Stockmeier, C.A., Mahajan, G.J., Konick, L.C., Overholser, J.C., Jurjus, G.J., Meltzer, H.Y., Uylings, H.B., Friedman, L., Rajkowska, G., 2004. Cellular changes in the postmortem hippocampus in major depression. *Biol. Psychiatry* 56, 640–650.

Stockmeier, C.A., Rajkowska, G., 2004. Cellular abnormalities in depression: evidence from postmortem brain tissue. *Dialogues Clin. Neurosci.* 6, 185.

Strack, S., Colbran, R.J., 1998. Autophosphorylation-dependent targeting of calcium/calmodulin-dependent protein kinase II by the NR2B subunit of the N-methyl-D-aspartate receptor. *J. Biol. Chem.* 273, 20689–20692.

Stroissnigg, H., Trancikova, 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. *Nat. Cell Biol.* 9, 1035.

Suddath, R.L., Casanova, M.F., Goldberg, T.E., Daniel, D.G., Kelsoe, J.R., Weinberger, D.R., others, 1989. Temporal lobe pathology in schizophrenia: a quantitative magnetic resonance imaging study. *Am J Psychiatry* 146, 464–472.

Sunanda, Rao, M.S., Raju, T.R., 1995. Effect of chronic restraint stress on dendritic spines and excrescences of hippocampal CA3 pyramidal neurons—a quantitative study. *Brain Res.* 694, 312–317. [https://doi.org/10.1016/0006-8993\(95\)00822-8](https://doi.org/10.1016/0006-8993(95)00822-8)

Sunico, C.R., González-Forero, D., Domínguez, G., García-Verdugo, J.M., 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. *J. Neurosci.* 30, 973–984.

Svitkina, T.M., Bulanova, E.A., Chaga, O.Y., Vignjevic, D.M., Kojima, S., Vasiliev, J.M., Borisy, G.G., 2003. Mechanism of filopodia initiation by reorganization of a dendritic network. *J Cell Biol* 160, 409–421.

Swanson, R.A., Chen, J., Graham, S.H., 1994. Glucose can fuel glutamate uptake in ischemic brain. *J. Cereb. Blood Flow Metab.* 14, 1–6.

Tada, T., Sheng, M., 2006. Molecular mechanisms of dendritic spine morphogenesis. *Curr. Opin. Neurobiol.* 16, 95–101.

Tahirovic, S., Bradke, F., 2009. Neuronal polarity. *Cold Spring Harb. Perspect. Biol.* 1, a001644.

Takano, T., Oberheim, N., Cotrina, M.L., Nedergaard, M., 2009. Astrocytes and ischemic injury. *Stroke J. Cereb. Circ.* 40, S8–12. <https://doi.org/10.1161/STROKEAHA.108.533166>

- Takei, N., Sasaoka, K., Inoue, K., Takahashi, M., Endo, Y., Hatanaka, H., 1997. Brain-Derived Neurotrophic Factor Increases the Stimulation-Evoked Release of Glutamate and the Levels of Exocytosis-Associated Proteins in Cultured Cortical Neurons from Embryonic Rats. *J. Neurochem.* 68, 370–375.
- 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. *Environ. Toxicol. Pharmacol.* 40, 199–205.
- Tanaka, E., Sabry, J., 1995. Making the connection: cytoskeletal rearrangements during growth cone guidance. *Cell* 83, 171–176.
- Tanaka, J., Toku, K., Zhang, B., Ishihara, K., Sakanaka, M., Maeda, N., 1999. Astrocytes prevent neuronal death induced by reactive oxygen and nitrogen species. *Glia* 28, 85–96.
- Tata, D.A., Marciano, V.A., Anderson, B.J., 2006. Synapse loss from chronically elevated glucocorticoids: Relationship to neuropil volume and cell number in hippocampal area CA3. *J. Comp. Neurol.* 498, 363–374. <https://doi.org/10.1002/cne.21071>
- Taylor, S., Anglin, J., Paode, P., Riggert, A., Olive, M., Conrad, C., 2014. Chronic stress may facilitate the recruitment of habit- and addiction-related neurocircuitries through neuronal restructuring of the striatum. *Neuroscience* 280, 231–242. <https://doi.org/10.1016/j.neuroscience.2014.09.029>
- Teng, F.Y.H., Tang, B.L., 2006. Axonal regeneration in adult CNS neurons—signaling molecules and pathways. *J. Neurochem.* 96, 1501–1508.
- Tessier-Lavigne, M., Goodman, C.S., 1996. The molecular biology of axon guidance. *Science* 274, 1123–1133.
- Thoenen, H., 1991. The changing scene of neurotrophic factors. *Trends Neurosci.* 14, 165–170.
- Thomas, C.G., Miller, A.J., Westbrook, G.L., 2006. Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J. Neurophysiol.* 95, 1727–1734.
- Toy, D., Namgung, U., 2013. Role of Glial Cells in Axonal Regeneration. *Exp. Neurobiol.* 22, 68–76. <https://doi.org/10.5607/en.2013.22.2.68>
- Tran, T.S., Rubio, M.E., Clem, R.L., Johnson, D., Case, L., Tessier-Lavigne, M., Huganir, R.L., Ginty, D.D., Kolodkin, A.L., 2009. Secreted semaphorins control spine distribution and morphogenesis in the postnatal CNS. *Nature* 462, 1065.
- Trivedi, M.H., Rush, A.J., Wisniewski, S.R., Nierenberg, A.A., Warden, D., Ritz, L., Norquist, G., Howland, R.H., Lebowitz, B., McGrath, P.J., Shores-Wilson, K., Biggs, M.M., Balasubramani, G.K., Fava, M., 2006. Evaluation of Outcomes With Citalopram for Depression Using Measurement-Based Care in STAR*D: Implications for Clinical Practice. *Am. J. Psychiatry* 163, 28–40. <https://doi.org/10.1176/appi.ajp.163.1.28>
- Tsai, M.-C., Huang, T.-L., 2016. Increased activities of both superoxide dismutase and catalase were indicators of acute depressive episodes in patients with major depressive disorder. *Psychiatry Res.* 235, 38–42. <https://doi.org/10.1016/j.psychres.2015.12.005>
- Tsai, M.J., Chang, Y.-F., Schwarcz, R., Brookes, N., 1996. Characterization of α -amino acid transport in cultured rat astrocytes. *Brain Res.* 741, 166–173.

- Tucker, K.L., Meyer, M., Barde, Y.-A., 2001. Neurotrophins are required for nerve growth during development. *Nat. Neurosci.* 4, 29.
- Tyler, W.J., Pozzo-Miller, L., 2003. Miniature synaptic transmission and BDNF modulate dendritic spine growth and form in rat CA1 neurones. *J. Physiol.* 553, 497–509.
- Ullian, E., Harris, B., Wu, A., Chan, J., Barres, B., 2004. Schwann cells and astrocytes induce synapse formation by spinal motor neurons in culture. *Mol. Cell. Neurosci.* 25, 241–251. <https://doi.org/10.1016/j.mcn.2003.10.011>
- Ullian, E.M., Christopherson, K.S., Barres, B.A., 2004. Role for glia in synaptogenesis. *Glia* 47, 209–216.
- Ullian, E.M., Sapperstein, S.K., Christopherson, K.S., Barres, B.A., 2001. Control of synapse number by glia. *Science* 291, 657–661.
- Varghese, F.P., Brown, E.S., 2001. The Hypothalamic-Pituitary-Adrenal Axis in Major Depressive Disorder: A Brief Primer for Primary Care Physicians. *Prim. Care Companion J. Clin. Psychiatry* 3, 151–155.
- Varoqueaux, F., Jamain, S., Brose, N., 2004. Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur. J. Cell Biol.* 83, 449–456.
- Verkhatsky, A., Olabarria, M., Noristani, H.N., Yeh, C.-Y., Rodriguez, J.J., 2010. Astrocytes in Alzheimer's disease. *Neurotherapeutics* 7, 399–412. <https://doi.org/10.1016/j.nurt.2010.05.017>
- Verstraelen, P., Pintelon, I., Nuydens, R., Cornelissen, F., Meert, T., Timmermans, J.-P., 2014. Pharmacological characterization of cultivated neuronal networks: relevance to synaptogenesis and synaptic connectivity. *Cell. Mol. Neurobiol.* 34, 757–776.
- Vicario-Abejón, C., Owens, D., McKay, R., Segal, M., 2002. Role of neurotrophins in central synapse formation and stabilization. *Nat. Rev. Neurosci.* 3, 965.
- Videbech, P., Ravnkilde, B., 2004. Hippocampal volume and depression: a meta-analysis of MRI studies. *Am. J. Psychiatry* 161, 1957–1966.
- Vignjevic, D., Kojima, S., Aratyn, Y., Danciu, O., Svitkina, T., Borisy, G.G., 2006. Role of fascin in filopodial protrusion. *J Cell Biol* 174, 863–875.
- Voleti, B., Navarria, A., Liu, R.-J., Banasr, M., Li, N., Terwilliger, R., Sanacora, G., Eid, T., Aghajanian, G., Duman, R.S., 2013. Scopolamine Rapidly Increases Mammalian Target of Rapamycin Complex 1 Signaling, Synaptogenesis, and Antidepressant Behavioral Responses. *Nov. Ther. Depress.* 74, 742–749. <https://doi.org/10.1016/j.biopsych.2013.04.025>
- Volianskis, A., France, G., Jensen, M.S., Bortolotto, Z.A., Jane, D.E., Collingridge, G.L., 2015. Long-term potentiation and the role of N-methyl-D-aspartate receptors. *Brain Res.* 1621, 5–16.
- Volke, V., Wegener, G., Bourin, M., Vasar, E., 2003. Antidepressant- and anxiolytic-like effects of selective neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole in mice. *Behav. Brain Res.* 140, 141–147. [https://doi.org/10.1016/S0166-4328\(02\)00312-1](https://doi.org/10.1016/S0166-4328(02)00312-1)
- Vyas, A., Mitra, R., Shankaranarayana Rao, B.S., Chattarji, S., 2002. Chronic Stress Induces Contrasting Patterns of Dendritic Remodeling in Hippocampal and Amygdaloid Neurons. *J. Neurosci.* 22, 6810.

- Vyas, A., Pillai, A.G., Chattarji, S., 2004. Recovery after chronic stress fails to reverse amygdaloid neuronal hypertrophy and enhanced anxiety-like behavior. *Neuroscience* 128, 667–673.
- Walter, M., Henning, A., Grimm, S., Schulte, R.F., Beck, J., Dydak, U., Schnepf, B., Boeker, H., Boesiger, P., Northoff, G., 2009. The relationship between aberrant neuronal activation in the pregenual anterior cingulate, altered glutamatergic metabolism, and anhedonia in major depression. *Arch. Gen. Psychiatry* 66, 478–486.
- Wang, C., Sadovova, N., Patterson, T.A., Zou, X., Fu, X., Hanig, J.P., Paule, M.G., Ali, S.F., Zhang, X., Slikker, W., 2008. Protective effects of 7-nitroindazole on ketamine-induced neurotoxicity in rat forebrain culture. *NeuroToxicology* 29, 613–620. <https://doi.org/10.1016/j.neuro.2008.03.007>
- Wang, D.-L., Qian, X.-D., Lin, Y.-H., Tian, B.-B., Liang, H.-Y., Chang, L., Wu, H.-Y., Zhu, D.-Y., Luo, C.-X., n.d. ZL006 promotes migration and differentiation of transplanted neural stem cells in male rats after stroke. *J. Neurosci. Res.* n/a-n/a. <https://doi.org/10.1002/jnr.24068>
- Wang, G., Dinkins, M., He, Q., Zhu, G., Poirier, C., Campbell, A., Mayer-Proschel, M., Bieberich, E., 2012. Astrocytes secrete exosomes enriched with proapoptotic ceramide and prostate apoptosis response 4 (PAR-4) potential mechanism of apoptosis induction in Alzheimer disease (AD). *J. Biol. Chem.* 287, 21384–21395.
- Wang, J., Hodes, G.E., Zhang, H., Zhang, S., Zhao, W., Golden, S.A., Bi, W., Menard, C., Kana, V., Leboeuf, M., 2018. Epigenetic modulation of inflammation and synaptic plasticity promotes resilience against stress in mice. *Nat. Commun.* 9, 477.
- Wang, L., Chang, X., She, L., Xu, D., Huang, W., Poo, M., 2015. Autocrine action of BDNF on dendrite development of adult-born hippocampal neurons. *J. Neurosci.* 35, 8384–8393.
- Wang, L.-L., Hao, S., Zhang, S., Guo, L.-J., Hu, C.-Y., Zhang, G., Gao, B., Zhao, J.-J., Jiang, Y., Tian, W.-G., 2017. PTEN/PI3K/AKT protein expression is related to clinicopathological features and prognosis in breast cancer with axillary lymph node metastases. *Hum. Pathol.* 61, 49–57.
- Wang, Q., Jie, W., Liu, J.-H., Yang, J.-M., Gao, T.-M., 2017. An astroglial basis of major depressive disorder? An overview. *Glia*.
- Wang, X.-D., Su, Y.-A., Wagner, K.V., Avrabos, C., Scharf, S.H., Hartmann, J., Wolf, M., Liebl, C., Kühne, C., Wurst, W., Holsboer, F., Eder, M., Deussing, J.M., Müller, M.B., Schmidt, M.V., 2013. Nectin-3 links CRHR1 signaling to stress-induced memory deficits and spine loss. *Nat. Neurosci.* 16, 706.
- Warner-Schmidt, J.L., Duman, R.S., 2006. Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. *Hippocampus* 16, 239–249.
- Warren, B.L., Sial, O.K., Alcantara, L.F., Greenwood, M.A., Brewer, J.S., Rozofsky, J.P., Parise, E.M., Bolaños-Guzmán, C.A., 2014. Altered gene expression and spine density in nucleus accumbens of adolescent and adult male mice exposed to emotional and physical stress. *Dev. Neurosci.* 36, 250–260. <https://doi.org/10.1159/000362875>
- Washbourne, P., Bennett, J.E., McAllister, A.K., 2002. Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nat. Neurosci.* 5, 751.
- Watanabe, Y., Gould, E., McEwen, B.S., 1992. Stress induces atrophy of apical dendrites of

- hippocampal CA3 pyramidal neurons. *Brain Res.* 588, 341–345. [https://doi.org/10.1016/0006-8993\(92\)91597-8](https://doi.org/10.1016/0006-8993(92)91597-8)
- Watkins, L.R., Maier, S.F., 2003. GLIA: A novel drug discovery target for clinical pain. *Nat Rev Drug Discov* 2, 973–985. <https://doi.org/10.1038/nrd1251>
- Wellman, C.L., 2001. Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. *Dev. Neurobiol.* 49, 245–253.
- Whitfield-Gabrieli, S., Ford, J.M., 2012. Default mode network activity and connectivity in psychopathology. *Annu. Rev. Clin. Psychol.* 8, 49–76.
- Willard, S.S., Koochekpour, S., 2013. Glutamate, glutamate receptors, and downstream signaling pathways. *Int. J. Biol. Sci.* 9, 948.
- Williams, E.J., Walsh, F.S., Doherty, P., 1994. The production of arachidonic acid can account for calcium channel activation in the second messenger pathway underlying neurite outgrowth stimulated by NCAM, N-cadherin, and L1. *J. Neurochem.* 62, 1231–1234.
- Williams, M.R., Hampton, T., Pearce, R.K., Hirsch, S.R., Ansorge, O., Thom, M., Maier, M., 2012. Astrocyte decrease in the subgenual cingulate and callosal genu in schizophrenia. *Eur. Arch. Psychiatry Clin. Neurosci.* 263, 41–52. <https://doi.org/10.1007/s00406-012-0328-5>
- Willner, P., 1997. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl.)* 134, 319–329.
- Willner, P., 1984. The validity of animal models of depression. *Psychopharmacology (Berl.)* 83, 1–16.
- Winston, C.N., Noël, A., Neustadtl, A., Parsadanian, M., Barton, D.J., Chellappa, D., Wilkins, T.E., Alikhani, A.D., Zapple, D.N., Villapol, S., Planel, E., Burns, M.P., 2016. Dendritic Spine Loss and Chronic White Matter Inflammation in a Mouse Model of Highly Repetitive Head Trauma. *Am. J. Pathol.* 186, 552–567. <https://doi.org/10.1016/j.ajpath.2015.11.006>
- Wu, H.-Q., Ungerstedt, U., Schwarcz, R., 1995. L- α -amino adipic acid as a regulator of kynurenic acid production in the hippocampus: a microdialysis study in freely moving rats. *Eur. J. Pharmacol.* 281, 55–61.
- Wu, Y.K., Fujishima, K., Kengaku, M., 2015. Differentiation of Apical and Basal Dendrites in Pyramidal Cells and Granule Cells in Dissociated Hippocampal Cultures. *PLOS ONE* 10, e0118482. <https://doi.org/10.1371/journal.pone.0118482>
- Yalcin, I., Aksu, F., Belzung, C., 2005. Effects of desipramine and tramadol in a chronic mild stress model in mice are altered by yohimbine but not by pindolol. *Eur. J. Pharmacol.* 514, 165–174. <https://doi.org/10.1016/j.ejphar.2005.03.029>
- Yan, x., Shi, z.f., Xu, l.x., Li, j.x., Wu, m., Wang, x.x., Jia, m., Dong, l.p., Yang, s.h., Yuan, f., 2017. Glutamate Impairs Mitochondria Aerobic Respiration Capacity and Enhances Glycolysis in Cultured Rat Astrocytes [WWW Document]. <https://doi.org/10.3967/bes2017.005>
- Yang, C., Ko, B., Hensley, C.T., Jiang, L., Wasti, A.T., Kim, J., Sudderth, J., Calvaruso, M.A., Lumata, L., Mitsche, M., 2014. Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol. Cell* 56, 414–424.

- Yang, C., Shirayama, Y., Zhang, J., Ren, Q., Yao, W., Ma, M., Dong, C., Hashimoto, K., 2015. R-ketamine: a rapid-onset and sustained antidepressant without psychotomimetic side effects. *Transl Psychiatry* 5, e632.
- Yasumatsu, N., Matsuzaki, M., Miyazaki, T., Noguchi, J., Kasai, H., 2008. Principles of Long-Term Dynamics of Dendritic Spines. *J. Neurosci.* 28, 13592–13608. <https://doi.org/10.1523/JNEUROSCI.0603-08.2008>
- Yau, S.-Y., Li, A., Tong, J.-B., Bostrom, C., Christie, B.R., Lee, T.M., So, K.-F., 2016. Chronic corticosterone administration reduces dendritic complexity in mature, but not young granule cells in the rat dentate gyrus. *Restor. Neurol. Neurosci.* 34, 849–857. <https://doi.org/10.3233/RNN-160662>
- Yi, E.-S., Oh, S., Lee, J., Leem, Y.-H., 2017. Chronic stress-induced dendritic reorganization and abundance of synaptosomal PKA-dependent CP-AMPA receptor in the basolateral amygdala in a mouse model of depression. *Biochem. Biophys. Res. Commun.* 486, 671–678. <https://doi.org/10.1016/j.bbrc.2017.03.093>
- Yildiz-Yesiloglu, A., Ankerst, D.P., 2006. Review of 1 H magnetic resonance spectroscopy findings in major depressive disorder: a meta-analysis. *Psychiatry Res. Neuroimaging* 147, 1–25.
- Yoshihara, Y., De Roo, M., Muller, D., 2009. Dendritic spine formation and stabilization. *Curr. Opin. Neurobiol.* <https://doi.org/10.1016/j.conb.2009.05.013>
- Young, P., Qiu, L., Wang, D., Zhao, S., Gross, J., Feng, G., 2008. Single-neuron labeling with inducible Cre-mediated knockout in transgenic mice. *Nat. Neurosci.* 11, 721–728.
- Yüksel, C., Öngür, D., 2010a. Magnetic Resonance Spectroscopy Studies of Glutamate-Related Abnormalities in Mood Disorders. *Glutamate Mood Disord.* 68, 785–794. <https://doi.org/10.1016/j.biopsych.2010.06.016>
- Yüksel, C., Öngür, D., 2010b. Magnetic Resonance Spectroscopy Studies of Glutamate-Related Abnormalities in Mood Disorders. *Biol. Psychiatry* 68, 785–794. <https://doi.org/10.1016/j.biopsych.2010.06.016>
- Yuste, R., Bonhoeffer, T., 2004. Genesis of dendritic spines: insights from ultrastructural and imaging studies. *Nat. Rev. Neurosci.* 5, 24.
- Zamanian, J.L., Xu, L., Foo, L.C., Nouri, N., Zhou, L., Giffard, R.G., Barres, B.A., 2012. Genomic analysis of reactive astrogliosis. *J. Neurosci.* 32, 6391–6410.
- Zarate, C.A., Singh, J.B., Carlson, P.J., Brutsche, N.E., Ameli, R., Luckenbaugh, D.A., Charney, D.S., Manji, H.K., 2006. A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch. Gen. Psychiatry* 63, 856–864.
- Zhang, W., Benson, D.L., 2001. Stages of synapse development defined by dependence on F-actin. *J. Neurosci.* 21, 5169–5181.
- Zhang, W.-N., Chang, S.-H., Guo, L.-Y., Zhang, K.-L., Wang, J., 2013. The neural correlates of reward-related processing in major depressive disorder: A meta-analysis of functional magnetic resonance imaging studies. *J. Affect. Disord.* 151, 531–539. <https://doi.org/10.1016/j.jad.2013.06.039>
- Zhao, C., Teng, E.M., Summers, R.G., Ming, G., Gage, F.H., 2006. Distinct Morphological Stages of Dentate Granule Neuron Maturation in the Adult Mouse Hippocampus. *J. Neurosci.*

26, 3–11. <https://doi.org/10.1523/JNEUROSCI.3648-05.2006>

Zhao, Y., Jiang, Y., Lv, W., Wang, Z., Lv, L., Wang, B., Liu, X., Liu, Y., Hu, Q., Sun, W., Xu, Q., Xin, H., Gu, Z., 2016. Dual targeted nanocarrier for brain ischemic stroke treatment. *J. Controlled Release* 233, 64–71. <https://doi.org/10.1016/j.jconrel.2016.04.038>

Zhou, H.F., Lee, L.H.-C., Lund, R.D., 1990. Timing and patterns of astrocyte migration from xenogeneic transplants of the cortex and corpus callosum. *J. Comp. Neurol.* 292, 320–330. <https://doi.org/10.1002/cne.902920213>

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. *Nat Med* 16, 1439–1443. <https://doi.org/10.1038/nm.2245>

Zhou, Y., Takahashi, E., Li, W., Halt, A., Wiltgen, B., Ehninger, D., Li, G.-D., Hell, J.W., Kennedy, M.B., Silva, A.J., 2007. Interactions between the NR2B receptor and CaMKII modulate synaptic plasticity and spatial learning. *J. Neurosci.* 27, 13843–13853.

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 Res.* 1114, 1–10.

Zipursky, R.B., Lim, K.O., Sullivan, E.V., Brown, B.W., Pfefferbaum, A., 1992. Widespread cerebral gray matter volume deficits in schizophrenia. *Arch. Gen. Psychiatry* 49, 195–205.

Zipursky, R.B., Seeman, M.V., Bury, A., Langevin, R., Wortzman, G., Katz, R., 1997. Deficits in gray matter volume are present in schizophrenia but not bipolar disorder. *Schizophr. Res.* 26, 85–92.

Ziv, N.E., Smith, S.J., 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17, 91–102.