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The effects of antidepressants on the phenotype of activated microglia and ischemia-injured cortical neurons

by

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DEDICATION

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ABSTRACT

Depression is one of the most common disorders appearing following a stroke and is also a major factor limiting recovery and rehabilitation in stroke patients. Several antidepressants have shown to have anti-inflammatory properties within the central nervous system (CNS). The major source of pro-inflammatory factors within the CNS is from activated microglia, the innate immune cells of the CNS. Antidepressants have been shown to promote midbrain and hippocampal neuronal survival following an ischemic insult and these effects are mediated through the anti-inflammatory effects on microglia, but the effects on cortical neuronal survival after this insult have yet to be investigated. The present study aimed to test and compare antidepressants from three distinct classes (tricylics, monoamine oxidase inhibitors, and selective serotonin reuptake inhibitors [SSRIs]) on the release of inflammatory factors and amino acids from activated microglia and to determine whether altering this release could affect cortical neuronal viability after an ischemic insult. Primary microglia were treated with 1 μg/ml LPS and/or 10 μM antidepressants, and the various factors released into the medium were assayed. Co-cultures consisting of microglia and primary cortical neurons were used to assess the effects of antidepressant-treated activated microglia on the viability of ischemia-injured neurons. Of the antidepressants tested, most decreased the release of the pro-inflammatory factors nitric oxide, tumor necrosis factor-alpha, and interleukin 1-beta from activated microglia. Fluoxetine and citalopram, the SSRIs, also decreased the release of the amino acids glutamate and D-serine from LPS-activated microglia. Injured cortical neurons co-cultured with LPS-activated microglia pre-treated with fluoxetine and citalopram showed greater survival compared to injured neurons co-cultured with untreated activated microglia. Studies using NMDA receptor antagonists demonstrated

that the release of glutamate and D-serine from microglia was a principal factor mediating cortical neuronal survival. In addition, we found that one possible mechanism behind the attenuation of microglial glutamate and D-serine release following fluoxetine treatment is through the induction of microglial apoptosis. Our results demonstrated for the first time that fluoxetine and citalopram decrease the release of glutamate and D-serine from LPS-activated microglia and this increases the survival of injured cortical neurons after co-culture. Fluoxetine was also shown to induce the apoptotic death of microglia.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine; serotonin
ACTH	adrenocorticotrophic hormone
AG	aminoguanidine
AIDS	acquired immunodeficiency syndrome
AIF	apoptosis inducing factor
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
ANOVA	analysis of variance
AP-1	activator protein 1
APAF-1	apoptotic protease activating factor 1
APC	antigen presenting cell
ATP	adenosine triphosphate
BAPTA	1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid
BBB	blood-brain barrier
BCL-2	B-cell lymphoma 2
BDNF	brain-derived neurotrophic factor
BSA	bovine albumin
C3	caspase 3
C8	caspase 8
CA1	*cornu ammonis* area 1
CA3	*cornu ammonis* area 3
cAMP	cyclic adenosine monophosphate
CC3	cleaved caspase 3

CC8	cleaved caspase 8
CCR2	c-c chemokine receptor 2
CD	cluster of differentiation
CNPase	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS	central nervous system
CO_2	carbon dioxide
CRE	cAMP/Ca^{2+}-response element
CREB	cAMP/Ca^{2+}-responsive element binding protein
CRH	corticotrophin releasing hormone
CYP	cytochrome P 450
DA	dopamine
DAB	diaminobenzindine
DAPI	4',6-diamidino-2-phenylindole
DIV	days *in vitro*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSM-IV	diagnostic and statistical manual of mental disorders-fourth edition
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immuonosorbant assay

FADD	fas-associated protein with death domain
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GC-MS	gas chromatography-mass spectrometry
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GLT-1	glutamate transporter 1
GR	glucocorticoid receptor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
Iba1	ionized calcium binding adapter molecule-1
IDO	indolamine 2,3-dioxygenase
IFN-α	interferon-alpha
IFN-β	interferon-beta
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor 1
IL-1β	interleukin-1 beta
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10

iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRF-3	interferon regulatory factor 3
L701	NMDA receptor glycine/D-serine site inhibitor (L701,324)
LBP	lipopolysaccharide-binding protein
LPS	lipopolysaccharide
MAG	myelin associated glycoprotein
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MAP-2	microtubule-associated protein 2
MAP kinase	mitogen-activated protein
MBP	myelin basic protein
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MCP1	monocyte chemotactic protein 1
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYD88	myeloid differentiation primary response gene 88
NA	noradrenaline; norephinephrine
NADPH	nicotinamide adenine dinucleotide phosphate
NDDI	norephinephrine-dopamine disinhibitor
NDRI	norephinephrine-dopamine reuptake inhibitor

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NPC	neural precursor cell
NSC	neural stem cell
OGD	oxygen-glucose deprivation
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PET	positron emission tomography
PDE	phosphodiesterase
PFBC	pentafluorobenzoyl chloride
PGE2	prostaglandin E2
PKA	protein kinase A
P/S	penicillin/streptomycin
PVDF	polyvinylidene fluoride
RIP	ribosome inactivating protein
SERT	serotonin transporter
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
SNRI	serotonin-norephinephrine reuptake inhibitor
SSRI	selective-serotonin reupake inhibitor
STS	staurosporine

SVCC	supraventricular corpus callosum
TAT	trans-activator of transcription
TCA	tricyclic antidepressant
TCP	tranylcypromine
TEEN	tris-EDTA-EGTA-NaCl
TGF-β	transforming growth factor-beta
TIR	toll/IL-1 receptor
TLR	toll-like receptor
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-alpha
TNFR	tumor necrosis factor receptor
TRADD	tumor necrosis factor receptor type 1-associated death domain
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain-containing adaptor-inducing interferon beta
TrkB	tropomyosin kinase B

hr	hour
min	minute
ml	milliliter
mg	milligram
mm	millimeter
μl	microliter
μg	microgram

μM	micromolar
μm	micrometer
ng	nanogram
nM	nanomolar
nm	nanometer
M	molar
N	normal
^{o}C	degrees Celsius
H_2O	water
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate dibasic
NaH_2PO_4	monobasic sodium phosphate
NaOH	sodium hydroxide

CHAPTER 1

Introduction to depression: Different hypotheses and the role of inflammation

1.1. Introduction to depression

The term "depression" has been in use in psychiatry and neuroscience for over 150 years. Before 1800, only a few elements of what is now called depression were part of what was then termed "melancholia". Traditional melancholia was described as a madness of the few; however, in the modern era, depression is a much broader concept, now including deficiencies in the basics of well-being and the capacity to function in society (Vestergaard 2010). Depression is described as a prevalent, highly debilitating mental disorder affecting up to 15% of the population at least once in their lifetime, accompanied with overbearing costs for society (Masi and Brovedani 2011). The World Health Organization global burden of disease study places depression among the 10 leading medical causes of disability in the world and second only to ischemic heart disease (Murray and Lopez 1997). The most widely used criteria for diagnosing depression are found in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), fourth edition revised by the American Psychiatric Association. The criteria for diagnosis include the following symptoms:

1. Depressed mood.
2. Markedly diminished interest or pleasure in all or almost all activities.
3. Significant (>5% body weight) weight loss or gain or increase or decrease in appetite.
4. Insomnia or hypersomnia.
5. Psychomotor agitation or retardation.
6. Fatigue or loss of energy.

7. Feelings of worthlessness or inappropriate guilt.
8. Diminished concentration or indecisiveness.
9. Recurrent thoughts of death or suicide.

A diagnosis of major depressive disorder requires a patient to display a minimum of five of the listed symptoms for at least two weeks, where one of the symptoms must be depressed mood or loss of interest (Choudhary et al. 2012). Although depressive symptoms are well defined, the mechanisms underlying the onset and progression of the disease are often at the center of much debate. Many hypotheses have been generated to elucidate the mechanisms underlying the onset and progression of depression; however, hypotheses focused on monoamines, the hypothalamic-pituitary-adrenal (HPA) axis, and neurogenesis are not sufficient to describe these mechanisms entirely. Recently, an alternative hypothesis has received considerable attention and attempts to associate the immune system and inflammation to the etiology of depression. The principles behind the inflammatory hypothesis aim to link the principles of other hypotheses to inflammation, rather than disproving them entirely. Here, I will review these other hypotheses and their limitations and explain the importance of inflammation in the etiology of depression.

1.2. Historical hypotheses of depression

1.2.1. The monoamine hypothesis

The monoamine hypothesis describes the onset of depression as a result of a functional deficiency in monoamine neurotransmitters at certain synapses in the brain; the focus is

primarily on 5-hydroxytryptamine (5-HT; serotonin) and noradrenaline (NA, norepinephrine). The antidepressant drugs used to date increase the functional availability of monoamines by various mechanisms such as blocking neurotransmitter reuptake into nerve terminals, blocking neurotransmitter auto-receptors, or inhibiting monoamine oxidase, an enzyme responsible for the oxidative breakdown of monoamines. There are various classes of antidepressants including:

1. Tricyclic antidepressants (TCAs)
2. Monoamine oxidase inhibitors (MAOIs)
3. Selective-serotonin reuptake inhibitors (SSRIs)
4. Serotonin-norephinephine reuptake inhibitors (SNRIs)
5. Norepinephrine-dopamine reuptake inhibitors (NDRIs)
6. Norepinephrine-dopamine disinhibitors (NDDIs)

1.2.2. Emergence of antidepressants

Antidepressants emerged from clinical trials in the early 1950s of two hydrazine derivatives, isoniazid and iproniazid, designed to treat turberculosis (Moncrieff 2008). Patients treated with these drugs showed a remarkable improvement in their mood. Iproniazid was introduced as the first antidepressant based on its surprising action in alleviating depressive symptoms; it was also discovered to inhibit monoamine oxidase (MAO), leading to the emergence of the monoamine hypothesis of depression and to the development of drugs termed MAOIs. However, MAO inhibiton by these early drugs produced adverse side effects in patients through the elevation in blood pressure after the

consumption of food containing certain amines. Iproniazid and most MAOIs irreversibly inhibit the MAO-A subtype of the enzyme; however, MAO-A is required for tyramine metabolism in the gut, thus preventing the passage of tyramine into the blood (Youdim and Weinstock 2004). Non-metabolized tyramine increases NA release from nerve terminals and leads to side effects ranging from headaches to hypertensive crisis. This side effect, termed the "cheese effect" (tyramine is present in high concentrations in certain cheeses), meant that patients on MAOIs had to be on a restricted diet. Later studies led to the development of moclobemide, a reversible MAO-A inhibitor which possessed antidepressant properties but did not produce the "cheese effect" (Bonnet 2003). The TCAs were developed serendipitously when imipramine was synthesized as an analogue of the antipsychotic chlorpromazine and was discovered to have unexpected clinical antidepressant effects; this finding lead to the development of other TCAs (Pletscher 1991). The MAOIs phenelzine and tranylcypromine, and the TCAs imipramine, amitriptyline, and nortriptyline are examples of antidepressants widely used up until the 1980s, a time where a new generation of drugs, the SSRIs, were made available. SSRIs are preferred over previous antidepressants due to the adverse side effects and potentially severe drug-drug and food-drug interactions with the MAOIs and TCAs (Pacher et al. 2001).

1.2.3. Monoamine hypothesis anomolies

The principles behind the monoamine hypothesis fail to explain the anomalies found in research and clinical trials. The time discrepancy between the onset of antidepressant treatment and the delayed clinical improvements observed in patients cannot be explained

adequately by the monoamine hypothesis. Depressed patients treated with antidepressants often show rapid elevations in extracellular monoamine levels, but clinical improvements in their behavior and mood most often do no surface until 2-3 weeks post-treatment, whereas the monoamine hypothesis would predict quick clinical improvements caused by the rapid elevations in the monoamines (Hindmarch 2001). The monoamine hypothesis cannot explain the effects of certain drugs that have antidepressant effects such as iprindole and tianeptine. Iprindole is a very weak inhibitor of monoamine reuptake and predominantly acts as an antagonist at 5-HT_2 receptors (De Ceballos et al. 1985) whereas tianeptine is a reuptake enhancer that is effective in removing 5-HT from the extracellular space (Mutlu et al. 2012). The monoamine hypothesis cannot explain how these drugs are effective antidepressants by decreasing serotonergic activity. Finally, the hypothesis cannot explain how certain drugs that enhance monoamine transmission within the brain (e.g. cocaine and amphetamine) are not effective antidepressants (Hindmarch 2001; Richelson 2003; Taylor et al. 2005; McCarthy et al. 2012; Padgett et al. 2012). Standing alone, the monoamine hypothesis is thus inadequate in explaining the etiology of depression.

1.2.4. The HPA axis hypothesis

A malfunction in the hypothalamic-pituitary-adrenal (HPA) axis can play an important role in the onset of depression, commonly after stress exposure. A brain structure that is well known for its involvement in depression is the hypothalamus. This small brain region consisting of several nuclei and located anterior to the third ventricle is considered to play a major role in the emergence and persistence of depressive symptoms. Indeed,

the hypothalamus, despite its limited size, is crucially involved in the regulation of autonomic and endocrine responses in order to meet physiological demands in maintaining internal homeostasis (Nesse 2000). The paraventricular nucleus of the hypothalamus is responsible for the synthesis and secretion of peptides including corticotrophin releasing hormone (CRH) and vasopressin (Engelmann et al. 2004). These peptides regulate the anterior lobe of the pituitary gland to stimulate the secretion of adrenocorticotrophic hormone (ACTH) (Engelmann et al. 2004). ACTH is secreted into the plasma and stimulates the adrenal cortices to produce glucocorticoids, primarily cortisol (Klavdieva 1996). Finally, glucocorticoids complete a negative feedback loop by inhibiting the production of CRH and ACTH from the hypothalamus and pituitary, respectively (Heim et al. 2008).

Blood cortisol levels are highly dependent on hypothalamic activity through the secretion of CRH and ACTH (Klavdieva 1996). Typically, blood cortisol levels are highest in the morning, gradually decline during the day, rise in the afternoon, and finally decline in the evening in healthy individuals (Reynolds 2012). Stressful stimuli can influence the hypothalamic secretion of CRH, leading to abnormally high levels of cortisol secreted by the adrenal gland. Abnormally high cortisol levels impair glucocorticoid feedback through decreasing the functioning of glucocorticoid receptors (GRs) of the hypothalamus and pituitary, resulting in hyperactive hypothalamic CRH release after stress (Heim et al. 2008). Increases in the hypothalamic-derived CRH and adrenal-derived cortisol have been observed in the cerebrospinal fluid and plasma of patients with depression and these levels have been shown to be correlated to symptom severity

(Merali et al. 2004; Burke et al. 2005). A meta-analysis study has shown that depressed patients are more likely to present higher plasma cortisol levels compared to healthy controls (Stetler and Miller 2011). From this perspective, depression can be conceptualized as impairment in the HPA negative feedback mechanisms in response to excessive stress.

Stressors are of many diverse types and stress can be defined in many different ways such as 'normal' and 'chronic' stress. A number of factors can increase the strength of a stressful stimulus experienced by an individual, including a lack of physical activity, aging, a disease or illness, and drug abuse, all leading to abnormally high blood cortisol levels (Goncharova et al. 2010; Lester et al. 2010; He et al. 2012). Chronic stress can be interpreted as the prolonged and repeated exposure to unavoidable aversive situations, leading to alterations in homeostasis. Another definition stems from an animal's state of threatened homeostasis where the disturbing forces are termed 'stressors', and the resulting coping strategies are termed 'adaptive responses' (Pacak and Palkovits 2001). In addition, types of stressors can differ depending on their social or physical nature. For instance, animal models of social stress include isolation, novel environments, crowding, novel cage mate, and dominance or social hierarchy (Kabbaj et al. 2001; Isgor et al. 2004; Schmidt et al. 2010; McCormick et al. 2012). Animal models of physical stress include the forced swim test, restraint, cold exposure, cage tilt, food or water deprivation, footshock, elevated platform exposure, and odor exposure (Lepsch et al. 2005; Toth et al. 2008; Tsoory et al. 2008; Jankord et al. 2011; Toledo-Rodriguez et al. 2012). All these stressors, social or physical, can stimulate the activity of the HPA axis.

The hypothalamus receives and sends projections to the amygdala and hippocampus, brain regions important in mediating emotional responses to stress and fear. These connections allow the subject to modulate their reaction to stress; however, an overactive HPA axis can be detrimental to associated brain regions. For instance, excessive levels of glucocorticoids produced by an overactive HPA axis can lead to hippocampal atrophy in humans and rodent models (Massart et al. 2012). This atrophy can markedly affect the ability of an individual or rodent to cope with and respond normally to stress.

Cross communication between monoamine neurotransmitters (dopamine, 5-HT, and NA) and the HPA axis has been shown to be important in mediating responses to stress. For instance, elevations in CRH levels down-regulate neuronal activity of the serotonergic dorsal raphe nuclei and the dendrites of the locus coeruleus, a region rich in noradrenergic neurons (Van Bockstaele et al. 1998; Kirby et al. 2000). Some drugs that can alter HPA axis function and activity have been shown to have antidepressant effects. For instance, CRH receptor antagonists have been reported to exert antidepressant activity in animal models (Louis et al. 2006). In addition, a glucocorticoid receptor inhibitor, mifepristone, is an effective treatment for depression, but only for severely depressed patients (Flores et al. 2006).

A problem with the majority of research focusing on the HPA axis is its application of experimental stress to induce depressed symptoms in rodent models. Severe stressors to which rodents are exposed in these studies do not parallel common stressors in humans,

especially in childhood. For instance, humans seldom receive inescapable electrical shocks similar to rodents in various experimental models (de Paula Soares et al. 2011; Smalheiser et al. 2011). Also, there is a lack of animal models that display behavioral symptoms resembling those of human subjects. In addition, contrary to the hypothesis, many patients diagnosed with depression show no evidence of HPA axis dysfunction (Belmaker and Agam 2008). Although altered activity of the HPA axis is one of the most commonly observed neuroendocrine abnormalities in patients suffering from depression, only 50% of depressed patients exhibit an up-regulation in cortisol levels (Bornstein et al. 2008). Therefore, HPA axis deregulation is also inadequate in fully explaining depressive etiology.

1.2.5. The neurogenesis hypotheses of depression

In recent years, hypotheses describing the concept of neurogenesis have concentrated on the long-term changes in signal transduction systems and gene expression in depression (Malki et al. 2012). Neurogenesis is described as the proliferation and differentiation of precursor cells into new neurons with characteristics indistinguishable from existing neurons (Toro and Deakin 2007). Adult neurogenesis is predominant in two regions of the brain, namely:

1. The subventricular zone, lining the lateral ventricles. Progenitor cells here differentiate into neurons and can migrate to the olfactory bulb.
2. The subgranular zone, located in the hippocampus within the dentate gyrus.

The region of interest of neurogenesis in depression is the hippocampus. The hippocampus is involved primarily in learning and memory in response to various stimuli including stressors. Studies have demonstrated that stress can play a major role in the regulation of postnatal hippocampal neurogenesis (Mirescu and Gould 2006). Postnatal hippocampal neurogenesis is attenuated by many of the same mediators that predispose individuals to depression and is stimulated by antidepressant treatments (Kempermann and Kronenberg 2003; Perera et al. 2008; Thomas and Peterson 2008). These observations have generated the hypothesis that, although reduced hippocampal neurogenesis may not itself produce depression, it may be an important factor in the progression of the disorder and stimulation of neurogenesis may be essential for effective antidepressant treatment (Evans et al. 2012). By disrupting neurogenesis, behavioral responses to antidepressants can be diminished (Santarelli et al. 2003). For instance, chronic treatment with the SSRI fluoxetine has been shown to accelerate the maturation of immature neurons in the hippocampus and improve behavioral symptoms in depressed-induced rodents; however, when neurogenesis is ablated through X-irradiation, fluoxetine's behavioral effects are inhibited (Wang et al. 2008).

There are links correlating neurogenesis to the HPA axis. In addition to inducing depressive-like behavior in rodents, glucocorticoids induce hippocampal neuronal cell death and reduce neurogenesis (Sapolsky 2001; Murray et al. 2008; David et al. 2009) and these effects can be attenuated by removing the adrenal glands in these rodents to decrease glucocorticoid levels (Gould et al. 1992; Tanapat et al. 1998). In addition, fluoxetine has been shown to restore the hippocampal regulation of the HPA axis under

chronic stress conditions, but only in the presence of intact neurogenesis and proper functioning of GRs (Surget et al. 2011). A further study shows that mice with decreased levels of GRs display higher levels of glucocorticoids and reduced hippocampal neurogenesis compared to mice with normal levels of these receptors (Kronenberg et al. 2009).

A potential gene critical in mediating neurogenesis is the gene for the neurotrophin brain-derived neurotrophic factor (BDNF) (Richelson 2003). BDNF induces dendritic outgrowth, synaptic formation, survival, growth, and differentiation of both existing and developing neurons. Overall, there is little evidence supporting the role of BDNF in depression onset, and more evidence supporting its role in disease progression (Adachi et al. 2008). Mediators considered to be significant in the onset of depression have been shown to suppress BDNF expression and, in turn, neurogenesis. Physical or social stressors decrease levels of BDNF in the hippocampus and prefrontal cortex in rodent models of depression (Krishnan and Nestler 2008; Castren and Rantamaki 2010). For instance, stress-elevated glucocorticoid levels suppress BDNF induced-neurogenesis via blocking MAP kinase signaling in developing cultured hippocampal neurons (Kunugi et al. 2012). In addition, post-mortem brains of depressed patients show reduced levels of BDNF (Duman and Monteggia 2006). Studies designed to measure BDNF levels in blood show reduced levels in the serum of depressed patients (Sen et al. 2008; Bocchio-Chiavetto et al. 2010). Furthermore, antidepressant treatment prevents hippocampal neuronal death by increasing levels of BDNF; however, this increase is reliant on chronic treatment with the drugs (Duman and Monteggia 2006; Castren and Rantamaki 2010). In

addition, elevations in BDNF mRNA, protein, and its cognate receptor tropomyosin kinase B (TrkB) have been reported after escitalopram (an SSRI) and desipramine (a TCA) treatment of rats (Kozisek et al. 2008). That study also found that BDNF mRNA increases promptly following treatments, but increases in protein were not observed until after 7 days of treatment. These findings provide a logical explanation behind the delayed clinical effects observed in patients following antidepressant treatment.

Another protein involved in long-term neuronal changes is the cAMP/Ca^{2+}-responsive element binding protein (CREB). CREB is a transcription factor for BDNF and increasing levels of BDNF result in feedback phosphorylation of CREB through the TrkB receptor, leading to the up-regulation of more BDNF (Hindmarch 2001). CREB is activated by kinase phosphorylation mediated primarily through G-protein coupled receptors. CREB enters cell nuclei and mediates gene expression by interacting with cAMP/Ca^{2+} response element (CRE) in the DNA. In addition to BDNF, CREB mediates the expression of many genes including the genes responsible for the synthesis of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis, and CRH, helping explain the changes observed in monoamine levels and HPA axis activity in depression (Malberg and Blendy 2005; Aguilera and Liu 2012).

Antidepressants have been shown to influence neuronal cell death and damage in the hippocampus of rodents at least in part by mediating BDNF and CREB expression (Haynes et al. 2004; Sen et al. 2008). Fluoxetine treatment has been shown to alleviate reduced neurogenesis observed in glucocorticoid-induced depressed rodents (David et al.

2009). In addition, chronic treatment with antidepressants increases the number of neuronal precursor cells and cell proliferation, and reduces hippocampal tissue loss in depressed patients (Colla et al. 2007; Boldrini et al. 2009). Chronic administration of various 5-HT and NA reuptake inhibitors up-regulates the levels of CREB and cAMP, a second messenger activator of CREB, in the rat hippocampus (Nibuya et al. 1996). In addition, inhibitors of cAMP phosphodiesterase, an enzyme responsible for the catabolism of cAMP, show antidepressant-like effects in clinical trials, further supporting a role for CREB and cAMP in depression (Duman et al. 1999). There are reports that support reduced neurogenesis as a greater factor in progressing depressive-like symptoms when compared to a deficiency in CREB activity. This support stems from observations of CREB-mutant mice; these mutants cannot form functional CREB but display reduced depressive-like behavior and increased hippocampal neurogenesis after antidepressant treatment (Dranovsky and Hen 2006). This antidepressant-induced neurogenesis is abolished when glucocorticoid levels are decreased, suggesting that glucocorticoid signaling is required for antidepressant effects on neurogenesis (Huang and Herbert 2006). Therefore, the balance between glucocorticoid levels and neurogenesis is important in mediating depression.

A number of studies present data that oppose the principles behind the neurogenesis hypothesis. A study has shown that mice with a localized knockout of hippocampal BDNF fail to acquire most depressive symptoms, including a loss of sensitivity to reward, overall locomotor activity, and fear-conditioning (Adachi et al. 2008). Furthermore, another study has shown that a selective loss of BDNF in the CA1 region of

the mouse hippocampus is inadequate to induce depressive-like symptoms (Zakharenko et al. 2003). The discrepancies between studies could be a product of selected knockdown paradigms and behavioral methodology. The knockout of BDNF expression in neurons and glia produce differential behavioral outcomes depending on whether the knockout is global or localized in the rodent hippocampus (Taliaz et al. 2010). However, as stated previously, evidence supports BDNF levels influencing disease progression rather than onset, and thus a BDNF knockout may not necessarily produce symptoms. Also, a study based on a learned helplessness model in mice shows conflicting results in the context of CREB functionality in depression. The down-regulation in forebrain-specific CREB has been shown to reduce depressive-like behavior, whereas over-expression increases depressive behavior (Sakai et al. 2002). CREB's contribution in depression may be dependent on regional specificity, rather than it being a global mechanism (Sakai et al. 2002). Finally, with all the support behind the neurogenesis hypothesis, it fails to explain antidepressant effects in other regions of the brain outside the hippocampus. For example, the prefrontal cortex is susceptible during the course of depression and benefits from antidepressant treatment; however, there is no evidence of neurogenesis in the region. Also, reduced neurogenesis in depression has yet to be consistently demonstrated in clinical populations where some studies, including data from human post-mortem brains, find no changes in neurogenesis in patients (Reif et al. 2006).

1.3. The inflammatory hypothesis

1.3.1. Introduction to glia

The mechanisms underlying the onset and progression of depression are often at the center of much debate. The focus of research concerning these mechanisms often center on neuronal cellular death and neurocircuitry. It is irrefutable that neurons are key targets that contribute to the symptoms, development, and progression in depression; however, it is becoming well established that glial cells are also key contributors. Glial cells are responsible for maintaining homeostasis in the central nervous system (CNS) by providing support and protection for neurons. There are several types of glial cells in the CNS, including astrocytes, oligodendrocytes, and microglia. Astrocytes are star shaped cells that perform a number of crucial functions within the brain and spinal cord including balancing the extracellular ion concentrations, supporting the blood-brain barrier, regulating glutamate uptake, providing necessary nutrients for neurons, and repairing tissue after an injury or insult (Kelly and Rose 2010; Cantrill et al. 2012; Lee et al. 2012). Oligodendrocytes are responsible for insulating neuronal axons with a myelin sheath and enhancing the speed of neurotransmission (Makinodan et al. 2012). However, researchers working on the inflammatory hypothesis have focused on microglia, the innate immune and sensor cells within the CNS.

1.3.2. History of microglia

The first scientist to call the innate immune cells of the CNS "microglia" was Pio Del Rio Hortega in 1919 (McGeer and McGeer 2011). Using the Golgi staining technique, Hortega identified microglia as small spidery cells that migrate to the brain in late embryonic life (McGeer and McGeer 2011). In development, cells arise from three primary germ layers termed the ectoderm, mesoderm, and endoderm. For decades, there

has been debate focused on microglial origin. Evidence suggests that microglia arise from the bone marrow, flexible tissue of mesodermal orgin. Peripheral monocytes, also from bone morrow, are indistinguishable from microglia, and thus it is thought that peripheral monocytes infiltrate the CNS during early development and become trapped within a structured blood-brain barrier (BBB), and as a result, reside in the CNS as resident microglia, whereas the remaining cells of the CNS arise from the ectoderm (Perry et al. 1985; Cuadros et al. 1992; Ling and Wong 1993). As resident cells of the CNS, microglia make up 20% of the total glial population (Kreutzberg 1995). From 1927 to 1932, Hortega performed brain lesion experiments to characterize microglial responses and found a plethora of microglia in the corpus collosum and other white matter areas (del Rio-Hortega 1937). After a falling out with his mentor Ramon y Cajal over publishing issues, Hortega set up labs in Madrid, Paris, Oxford, and finally Bueno Aires to pursue his research, eventually succumbing to cancer in 1945 (McGeer and McGeer 2011). After his death, Hortega's interpretations and conclusions on his findings were both challenged and supported by many scientists.

1.3.3. Functional morphology of microglia

The first scientist to provide support of Hortega's claims was Wilder Penfield as he described the various morphologies of microglia, from resting to reactive (Penfield 1925). Penfield characterized microglia as dynamic cells that can undergo a number of structural changes based on their location and environment. The dynamic nature of microglia allows these cells to carry out immunological functions within the CNS without the need for macrophage replacement from the periphery. In the healthy adult brain,

microglia are described as having numerous branches protruding from rod shaped cell bodies and are often termed 'ramified' or 'resting' (Davalos et al. 2005; Nimmerjahn et al. 2005). The term 'resting' microglia is often a misnomer since microglia are never resting *per se*. The branches of microglia are in constant movement in order to survey for minor fluctuations, foreign particles, or injuries in the environment. Ramified microglia express low numbers of antigens, are immunologically silent, and display low phagocytotic activity, but these features become abundant when microglia become reactive or activated (**Figure 1-1**).

Resting microglia undergo morphological and functional changes and become activated following the detection of stimuli by their protruding branches or soma. Activated microglia undergo several morphological changes compared to resting microglia including the retraction of branches, enlargement of cell bodies, and the uptake and expression of immunological molecules such as HLA-DR1, a protein apart of the major histocompatibilty complex (MHC II) family (Murthy and Stern 1997). MHC II proteins are expressed primarily on antigen-presenting cells (APCs) including activated microglia and peripheral macrophages. Following the uptake of an antigen, primarily mediated through endocytosis, the antigen forms a complex with a MHC II protein within these cells. This complex is then transferred to the surface of the cell and presented to peripheral immune cells. This allows for the recruitment of peripheral immune cells such as T-cells, macrophages, neutrophils, and dendritic cells into the CNS. Alternatively, activated microglia increase their numbers through rapid proliferation; therefore, they do

not require recruitment of peripheral immune cells in the early stages of a disturbance (Graeber 2010).

The functional phenotypes of activated microglia are diverse depending on the stimuli used to activate the cells, the severity of the stimuli, region of interest within the CNS, and developmental age (Lai and Todd 2008). Therefore, to state that microglia are simply 'activated' is an over-generalization. Microglia, based on the activation profiles of peripheral macrophages, are known to exist in two main classes of activation, M1 and M2 (**Figure 1-2**). M1 activation results in the production of pro-inflammatory mediators after stimulation with activators such as lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) whereas M2 activation results in the release of anti-inflammatory and trophic factors after stimulation with anti-inflammatory cytokines and growth factors, such as interleukin-4 (IL-4), IL-10, and transforming growth factor-beta (TGF-β) (Ransohoff and Perry 2009). Activation profiles of microglia are mediated by a number of receptors on the surface or within a cell including TNF-α, purinergic, glutamate, interleukin, and toll-like receptors. These receptors respond to a specific stimulus and instigate a signaling cascade to produce a variety of outcomes. For instance, toll-like receptors (TLRs) are a family of receptors that identify pathogen-derived molecules such as LPS, proteoglycans, and viruses (van Noort and Bsibsi 2009). More specifically, LPS, a bacterial endotoxin, stimulates a class of TLRs (TLR4) to release a plethora of inflammatory factors and cytokines including interleukin 1-beta (IL-1β) (Kim et al. 2004; Lai and Todd 2008), tumor necrosis factor-alpha (TNF-α) (Jung et al. 2007; Park et al. 2007), and nitric oxide (NO) (Bi et al. 2005; Wu et al. 2007). Cytokines are small signaling proteins that have a

number of roles including mediating chemotaxis, regulating cellular death, and stimulating further cytokine production, all in an effort to modulate an inflammatory response. In addition to pro-inflammatory cytokines, activated microglia release anti-inflammatory cytokines, trophic factors, and chemokines including interleukin-4 (IL-4), IL-10, glial-derived neurotrophic factor (GDNF), BDNF, and monocyte chemotactic protein 1 (MCP1). Microglial-derived factors may target nearby cells or the same cell that produced and released the factor, termed paracrine and autocrine signaling, respectively. Depending on the stimulus, microglia have detrimental or beneficial effects towards both their own survival and the survival of proximal cells. For instance, *in vitro* studies show that the severity of neuronal injury in different brain regions determines the release of inflammatory and trophic mediators from microglia (Lai and Todd 2008). In addition, the same cytokine can have detrimental or benefical outcomes depending on the type of receptor it stimulates. For instance, cells may express two classes of TNF-α receptors TNFR1 and TNFR2; TNFR1 stimulation is linked to cellular death, whereas TNFR2 stimulation is linked to cell survival (Lambertsen et al. 2009).

Activated microglia are highly prevalent in various neurodegenerative diseases and CNS injuries (Ling et al. 2001), and mediators released from activated microglia can proceed to affect the activity of proximal neurons to produce symptoms such as neuropathic pain. For instance, Beggs and colleagues (2012) elegantly reviewed the cross communication between microglia and neurons in the spinal cord in models of neuropathic pain. In brief, various mediators including adenosine triphosphate (ATP) and the chemokine MCP-1 can be released from damaged peripheral neurons extending into the spinal cord. MCP-1

interacts with its cognate receptor CCR2 on microglia to induce several changes, including upregulation in the expression of the ATP receptor, P2X4 (Zhang et al. 2007; Toyomitsu et al. 2012). ATP released from damaged primary sensory neurons and neurons in the dorsal horn can bind to P2X4 receptors, stimulating the release of BDNF from microglia. In turn, BDNF increases the concentration of intracellular chloride within neurons of the spinal dorsal horn, thus disrupting the transmission of nociception and pain. This is merely one simplified example of how microglia and neurons may interact to produce symptoms such as neuropathic pain after an injury.

During their activated state, microglia can become what is termed 'ameoboid', characterized as spherical, large, and phagocytic (Graeber 2010). Phagocytic microglia migrate to a site of injury and engulf foreign material or cellular debris in order to prevent further damage to surrounding healthy cells. Microglia also engulf antigens through phagocytosis in order to become APCs. Phagocytic microglia are highly prevalent in the developing brain for synaptic pruning and rewiring (Tremblay et al. 2010; Paolicelli et al. 2011). During development, neurons make far more synaptic connections than are maintained in the mature brain where highly active neurons and synapses are maintained, whereas less active synapses are eliminated (Huberman et al. 2008) and in the absence of phagocytic microglia, this brain development through synaptic pruning does not occur (Tremblay et al. 2011). Microglia can manage their cell number by apoptotic cellular death in the CNS.

1.3.4. Inflammation, microglia, and depression

Inflammation often accompanies the onset and progression of neuropsychiatric disorders including depression, and microglia are the primary source of inflammatory mediators in the CNS. As mentioned earlier, microglia can enhance the release of inflammatory cytokines in response to activation (Loftis et al. 2010). Several factors determine the beneficial or detrimental outcome in depression after inflammatory release. Such factors include the time course and severity of the disorder, severity of injury, the type of molecule elevated, the amount of molecule elevated, and the age of the affected patient. For instance, aging has been correlated with the over-activation of microglia and increased production of pro-inflammatory cytokines in mood disorders (Sparkman and Johnson 2008).

The inflammatory hypothesis describes the onset and progression in depression as the result of an exaggerated inflammatory response. The principles behind the inflammatory hypothesis attempt to link inflammation to the biochemical alterations described in the other previously discussed hypotheses; therefore, this hypothesis is not designed to reject the principles behind other hypotheses, but instead designed to explain their anomalies and alterations in depression. Here, we will discuss these associations between previous hypotheses and inflammation.

1.4. The association between inflammation and historical hypotheses of depression

1.4.1. Inflammatory hypothesis link to monoamine levels

Microglia produce cytokines that influence monoamine levels within the CNS. Pro-inflammatory cytokines including interferons (IFNα and IFNγ), IL-1, and TNF-α induce indolamine 2,3-dioxygenase (IDO), an enzyme that metabolizes tryptophan to produce kynurenine (Guillemin et al. 2005). Tryptophan is the amino acid precursor for serotonin; therefore, an increase in IDO activity leads to a reduction in tryptophan for its conversion to serotonin. IDO's byproduct, kynurenine, is further metabolized to produce the neuroactive byproducts quinolinic acid and hydroxykynurenine (Corona et al. 2012). Quinolinic acid is associated with neurotoxic effects including lipid peroxidation and agonism of glutamate receptors, leading to neuronal death (Dantzer et al. 2011; Haroon et al. 2012). Quinolinic acid has been shown to be elevated in the cerebrospinal fluid of patients with depression (Steiner et al. 2011). Resulting depletions in brain tryptophan levels have been shown to cause transient decreases in mood (Harrison et al. 2002; Yatham et al. 2012). However, it is likely that other pathways exist that limit typtophan availability for serotonin synthesis. For instance, one study found no alterations in the levels of plasma IDO mRNA and kynurenine in depressed patients compared to healthy controls; however, tryptophan levels were reduced, accompanied by increases in levels of IFNγ and IL-6 cytokine levels (Hughes et al. 2012). In addition, this study did not observe increases in TNF-α or IL-1β, cytokines known to be inducers of IDO. Therefore, cytokines may activate alternative pathways independent of IDO and the pathway activated may depend on the type of cytokine elevated. Additional research focusing on tryptophan metabolism in depression must be done to elucidate these mechanisms.

Reduced 5-HT functional availability in depressed patients can be a result of increased expression of the serotonin transporter (SERT). Expression of the SERT has been shown to be associated with cytokine levels. For instance, an animal model of depression involving the *in vivo* administration of a HIV-1 protein (Tat) has been shown to induce the microglial release of TNF-α and IL-6 accompanied by increases in SERT expression; interestingly, inhibition of p38 MAP kinase attenuates the increases in cytokines and SERT expression after Tat administration (Zhu et al. 2010; Fu et al. 2011). Therefore, the link between cytokines and SERT expression can lead to a decrease in serotonin transmission in depression by making less serotonin available in the synaptic cleft. These findings should be interpreted with caution since expression levels do not necessarily reflect activity levels of a transporter.

The inflammatory hypothesis may help explain the anomalies of the monoamine hypothesis, including the time delay between antidepressant treatment and clinical outcomes. The monoamines – 5-HT (serotonin), dopamine, NA, and epinephrine – do not cross the BBB (Hinz et al. 2011); therefore, a limited number of monoamine neurotransmitter molecules exist in the CNS and these levels are modulated through various synthesis and breakdown pathways. Antidepressant drugs that inhibit reuptake do not directly increase the total number of active monoamine molecules in the CNS, but that number can be increased through the administration of amino acid precursors of monoamines, e.g. tryptophan (Hinz et al. 2011). The initial elevations in extracellular monoamine levels caused by antidepressants may not be sufficient to induce positive effects in depressed patients, but these precursors can cross the BBB and are metabolized

in the CNS into new monoamine molecules. Therefore, antidepressant treatment utilized to increase the short term serotonin availability at synapses may not be efficient at alleviating depressive symptoms and the delayed outcomes may stem from the antidepressant effects on inflammation to increase tryptophan availability for serotonin synthesis (McGrath et al. 2006).

1.4.2. Inflammatory hypothesis link to the HPA axis

In depressed patients, microglial-derived cytokines have been linked extensively to abnormalities in the HPA axis. Cytokines induce the release of CRH, ACTH, and glucocorticoids, primarily cortisol (Pariante and Miller 2001). Excessive cortisol levels then desensitize glucocorticoid receptors expressed in the hypothalamus, pituitary, and hippocampus, consequently altering normal feedback inhibition (Pariante and Miller 2001). Physiological levels of cortisol have been shown to inhibit the production of pro-inflammatory cytokines through glucocorticoid receptors; however, following stress exposure, cortisol-induced inhibition in cytokine production is lost due to a reduced activity of these receptors (Pucak and Kaplin 2005). In turn, pro-inflammatory cytokines enhance HPA axis activity (Antonijevic 2006; Miura et al. 2008). Cytokines have been shown to disrupt glucocorticoid receptor translocation from the cytoplasm to the nucleus and inhibit receptor binding to DNA, thereby disrupting negative feedback regulation (Pace et al. 2007). In addition, transforming growth factor-beta (TGF-β), an anti-inflammatory cytokine, has been shown to decrease depressive symptoms by inhibiting HPA axis activity; in accordance, it has been shown that depressed patients have reduced levels of TGF-β (Musil et al. 2011). Levels of cytokines and cortisol may not always

correlate with one another in depression. Cytokine levels seem to be more consistently elevated than cortisol in depressed patients. For instance, in response to LPS following dexamethasome administration, the majority of depressed subjects responded to signs of glucocorticoid resistance accompanied with increases in cytokine levels; however, glucocorticoid resistance was found in non-depressed patients, but increases in cytokine levels were absent (Vedder et al. 2007). Furthermore, a study investigating the plasma levels of IL-6 and cortisol in cancer patients comorbid with depression found that patients had increased plasma levels of IL-6; unexpectedly, cortisol levels were reduced rather than increased (Jehn et al. 2010). Another study investigated the plasma and serum levels of cortisol, IL-1β, and IL-6 in depressed patients before and after omega 3/fluoxetine treatment and they found that at 8 weeks post treatment there were no positive responses to treatment and no changes in cytokine levels, but a significant reduction in plasma cortisol levels (Jazayeri et al. 2010). In addition, another study found that 20 weeks into SSRI treatment, the psychiatric scales of patients indicated a return of depressive episodes paralleled with increases in IL-2 and IL-1β levels in the absence of changes in plasma cortisol levels (Hernandez et al. 2008). Therefore, cytokine levels may be more important than cortisol in mediating depressive symptoms.

Depressed patients often show altered levels of serotonin receptors, notably 5HT1A and 5HT2A. Both 5HT1A and 5HT2A receptors are G-protein coupled and mediate inhibitory and excitatory neurotransmission, respectively, and are found both pre- and post-synaptically. These receptors modulate a number of behaviors often affected in depression including anxiety, appetite, memory, mood, and sleep (Parks et al. 1998;

Yasuno et al. 2003; Ogren et al. 2008). By mediating glucocorticoid levels, cytokines regulate the expression of serotonin receptors, notably 5HT1A and 5HT2A (Leonard 2006). Cytokine-induced increases in cortisol, followed by alterations in serotonin receptor expression can be another mechanism by which the immune system influences depressive symptoms.

Evidence suggests microglial activation is prevalent in animal models of stress-induced depression (Blandino et al. 2009; O'Mahony et al. 2009). Animal paradigms show increases in ionized calcium binding adaptor molecule-1 (Iba1) in various brain regions susceptible to stress, including the amygdala, hypothalamus, hippocampus and prefrontal cortex (Tynan et al. 2010; Hinwood et al. 2012). Increases in Iba1 levels are an indicator of microglial reactivity or activation. It has been reported that rodents exposed to repeated stress show increases in the microglial-derived inflammatory molecules IL-1β and prostaglandin E2 (PGE2) in the medial prefrontal cortex, a region associated with emotional and cognitive changes in depression (Furuyashiki 2012). These researchers also noted that purinergic signaling through the P2X7 receptor can trigger PGE2 and IL-1β production from microglia in depression (Furuyashiki 2012). A histological study of postmortem brains from subjects diagnosed with depression showed increases in surface markers for microglial activation in the hippocampus and prefrontal cortex (Bayer et al. 1999). Recently, developments in positron emission tomography (PET) have allowed investigators to observe activated microglia in depressed patients in real time. PET scanning is a technique that detects emitted rays from an injected tracer coupled to a biologically active molecule. During activation, microglia increase the expression of a

mitochondrial translocator protein, and this protein binds to the PET-tracer [^{11}C]-PK11195 (Cosenza-Nashat et al. 2009). In depressed patients, PET scans show elevated levels of microglial activation in the hippocampus (Doorduin et al. 2009). In addition, it has been shown that stress-induced anhedonia (the loss in pleasure) in rodents was attenuated by the administration of the anti-inflammatory drug minocycline, an inhibitor of microglial activation (Tynan et al. 2010).

1.4.3. Inflammatory hypothesis link to neurogenesis

Under physiological conditions, microglial-derived cytokines enhance neurogenesis and contribute to normal cognitive functioning by providing trophic support to neurons (Goshen et al. 2007). However, excessive cytokine levels decrease the trophic support to neurons, reduce neurogenesis, increase oxidative stress, and sever neuronal-glial interactions (Wu et al. 2007; Koo and Duman 2008). For instance, excessive IL-1 levels are associated with deficits in cognition and neurogenesis; however, these deficits can be prevented through the administration of an IL-1 receptor antagonist into the hippocampus (Goshen et al. 2008). In addition, a deficit in neurogenesis is prevented when neural precursor cells are transplanted into the hippocampus and secrete an IL-1 receptor antagonist (Ben Menachem-Zidon et al. 2008). Cytokines can also affect the fate of neural precursor cells (NPCs) within the brain. Green et al. (2012) assessed the effect of IL-1β released from activated microglia on the proliferation and differentiation of embryonic rat hippocampal NPCs *in vitro* and concluded that IL-1β reduced the percentage of newly born neurons while increasing the percentage of glial cells, including astrocytes and oligodendrocytes, derived from NPCs. This shift to a greater percentage

of glial cells can lead to a further increase in cytokine levels, and decreased neurogenesis. Another study revealed that stress could produce depressive-like behaviors by activating NF-kappaB signaling and decreasing the proliferation of neural stem-like cells (Koo et al. 2010). NF-kappaB is a predominant protein complex involved in the production of cytokines. Cytokines are not only important in attenuating neurogenesis but they may also interfere with antidepressant treatment. For instance, fluoxetine has been shown to increase hippocampal neurogenesis in depressed rats; however, pretreatment with a high concentration of LPS to mimic excessive cytokine release attenuated the neurogenic actions of fluoxetine (Wang et al. 2011).

Evidence suggests an inverse correlation between cytokines and growth factors in depression. For instance, depressive symptoms can be induced in mice by exposing them to social defeat for 21 days (Gomez-Lazaro et al. 2011). This study found that defeated mice expressed greater levels of TNF-α and IL-6 and decreased levels in hippocampal BDNF. Concurring with these findings, depressed patients show decreased serum levels of BDNF and increased levels of IL-6 (Yoshimura et al. 2010). Intra-cerebral administration of growth factors in a rodent model of depression influences the brain and plasma levels of cytokines. For instance, in a LPS-induced rodent model of depression, insulin-like growth factor 1 (IGF-1) was shown to attenuate depressive symptoms, including reduced mobility in a forced swim test (Park et al. 2011). In addition to these findings, an increase in BDNF expression along with decreases in cytokine levels and IDO activity were observed after IGF-1 administration in rodents. However, it is important to take into account comorbid disorders that may accompany depression. For

instance, the biochemical basis and the response to antidepressants can differ between depressed patients diagnosed with Parkinson's and patients diagnosed solely for depression; after citalopram treatment patients with sole depression show increases in BDNF plasma levels and decreases in IL-6 levels whereas comorbid patients show only the reduction in IL-6 levels (Palhagen et al. 2010). Overall, the influence of cytokines on trophic support and neurogenesis appears to be a key mediator in depressive etiology.

1.4.4. Inflammatory hypothesis and glutamate excitotoxicity

Evidence suggests that cytokines can mediate depressive symptoms by inducing glutamate excitotoxicity (Piani et al. 1991). Cytokines induce glutamate release from microglia and astrocytes and reduce the uptake of glutamate by altering the expression of glutamate transporters (Tilleux and Hermans 2007; Ida et al. 2008). For instance, the cytokines TNF-α and IL-1 can modify extracellular glutamate levels in the CNS by reducing the uptake of glutamate by astrocytes (Hu et al. 2000; Carmen et al. 2009). Increases in extrasynaptic glutamate concentrations over-activate NMDA receptors in various regions of the brain (McCarthy et al. 2012). To support this view, the NMDA receptor antagonist ketamine has been shown clinically to have rapid antidepressant properties (Zarate et al. 2006; Pittenger et al. 2007). In addition, various drugs that have no direct effects on the degradation or uptake of monoamines but have antidepressant properties have been shown to decrease glutamate release in various regions of the brain. For instance, a study using stress-exposed rats found antidepressant effects and inhibition of glutamate release in the amygdala after treatment with the 5-HT_2 antagonists tianeptine and agomelatine (Reznikov et al. 2007). Furthermore, inflammatory cytokines affect the

function and expression of the astrocytic enzyme glutamine synthetase, an enzyme that catalyzes metabolism of glutamate into glutamine. Post-mortem studies indicate that brain glutamine synthetase function is suppressed in mood disorders (Kalkman 2011). Reduced functioning of glutamine synthetase can cause greater concentrations of glutamate and excitotoxic effects within the CNS. Finally, as stated earlier, pro-inflammatory cytokines induce indoleamine 2,3-dioxygenase (IDO) activity and increase the production of quinolinic acid, a potentially toxic agonist of the NMDA glutamate receptor. Thus, excessive quniolinic acid production is another means by which cytokines can cause excitotoxicity through glutamate receptors (Obrenovitch and Urenjak 2003).

1.5. Effects of antidepressants on microglia

Antidepressants have been shown to inhibit the release of inflammatory mediators from microglia. For instance, a study by Hashioka et al. (2007) reported that impramine (TCA), fluvoxamine (SSRI), and reboxetine (SNRI) inhibit the production of NO and IL-6 from cultured microglia after IFN-γ activation. The co-administration of inhibitors of cAMP or PKA with the antidepressants reversed these inhibitory effects on IL-6 and NO release. This result suggests an interaction between antidepressants and microglial inflammatory release. Selected antidepressants increase microglial cAMP levels and decrease cytokine expression. A probable target for antidepressants in microglia is phosphodiesterase (PDE), an enzyme that metabolizes cAMP. PDE activity can be altered during the course of depression and antidepressant treatment (Wong et al. 2006). By inhibiting PDE activity in microglia, antidepressants increase cAMP levels.

McMullan et al. (2012) showed that in addition to cytokines cAMP levels mediate glutamate release from LPS-activated microglia. However, to my knowledge, no studies have shown if antidepressants can influence glutamate release from activated microglia.

Further studies have shown that amitriptyline (TCA) and its active metabolite nortriptyline reduce IL-1β and TNF-α release from LPS-activated microglial cultures (Obuchowicz et al. 2006). The importance of inhibiting the release of IL-1β and TNF-α is that both cytokines are potent inducers of apoptosis in neurons (Ma et al. 2003). Thus, by inhibiting the release of IL-1β and TNF-α from microglia, antidepressants can have anti-apoptotic effects on neurons. In support of this view, Huang et al. (2007) reported that desipramine (TCA) inhibits the LPS-induced apoptosis of hippocampal-derived adult NSCs; desipramine increases the expression of Bcl-2, an anti-apoptotic protein and neuronal cell death repressor. In addition, this group found decreases in IL-1β, IL-6, and TNF-α levels in the culture medium of LPS-treated NSCs following desipramine treatment. The group failed to clarify if desipramine's effects on apoptosis are neuron-centric since they used undifferentiated cells.

The mechanism behind antidepressant attenuation of microglial cytokine release is highly debated. Cytokine release may be disrupted through impaired transcription, defective translation, or a malfunctioning release system. Cytokines are released through classical secretion defined by the packaging of molecules within secretory vesicles in the Golgi for storage and then secreted by receptor-mediated mechanisms termed 'regulated exocytosis' (Jolly and Sattentau 2007) or released rapidly through recycling smaller

vesicles termed "constituitive exocytosis' (Stow et al. 2009). Obuchowicz et al. (2006) showed that amitriptyline and nortriptyline did not induce any changes in the mRNA levels of IL-1β or TNF-α; however, cytokine release was attenuated. Furthermore, amitriptyline is a potent cell inhibitor of the influx of sodium and calcium, which are both required by the machinery responsible for the release of cytokines (Deffois et al. 1996). These observations support the idea that antidepressants reduce cytokine release through disrupting the machinery involved in release rather than transcriptional means. On the other hand, mRNA levels of cytokines in activated microglia can be altered by antidepressant treatment. For instance, fluoxetine (SSRI) attenuates the mRNA levels of TNF-α and IL-1β by inhibiting the phosphorylation and nuclear translocation of the NF-κB subunit in LPS-activated microglia (Liu et al. 2011). Therefore, the exact mechanism underlying antidepressant effects on cytokine expression and release may depend on the antidepressant.

1.6. Inflammatory hypothesis anomolies

There are studies that claim to contradict the inflammatory hypothesis. Some of these studies have failed to observe increases in the expression of several cytokines including IL-1β, IL-6, and TNF-α, in stressed or depressed patients (Rothermundt et al. 2001; Deakin 2003). However, these studies failed to measure other cytokines highly associated with depression, such as the interferons. It is possible, depending on the severity of the stressor, that cytokine levels are altered only moderately. Another contradictory study investigated the effects of fluoxetine on cytokine expression in microglia. The group reported that fluoxetine increased IL-1β and TNF-α mRNA levels,

NO release, and expression of iNOS in cultured microglia (Ha et al. 2006). However, the study failed to show increases in IL-1β and TNF-α protein release from microglia. Nonetheless, these results raise numerous questions about the inflammatory hypothesis. Fluoxetine is a widely used antidepressant and the treatment of depression with this drug may increase the expression of cytokines and NO in other regions of the brain that contain physiologically resting microglia. However, Cardenas et al. (2005) have indicated that NO has a dual role in neurogenesis in depression. The effects of NO on neurogenesis may depend on the type of nitric oxide synthase (NOS) used for its synthesis. *In vivo* studies have shown that NO produced from inducible and endothelial isoforms of NOS stimulate neurogenesis (Reif et al. 2004) whereas NO produced from the neuronal isoform reduces neurogenesis (Moreno-Lopez et al. 2004). The predominant form of NOS in microglia is the inducible isoform. Therefore, fluoxetine-induced NO release by microglia may be beneficial for neurogenesis. However, it is possible that the over-expression of NO from activated microglia stimulates the production of NO from neurons. Neuronal-derived NO may possibly overshadow the beneficial effects of resting microglial-derived NO on neurogenesis. In addition, fluoxetine treatment does not elevate NO levels to the extent seen with the microglial activators LPS and IFN-γ. Furthermore, the fluoxetine study by Tynan et al. (2012) investigated the drug's effects on resting microglial cultures, and fluoxetine has been shown to attenuate NO release from the activated state. These observations support the importance of NO levels in the CNS. High levels of NO released by activated microglia may result in toxicity and low to moderate levels of NO released from resting microglia

may promote neurogenesis. However, further studies are necessary to investigate the role of NO in depression and antidepressant treatment.

1.7. Co-morbidity of stroke and depression

Sir William Osler, the father of modern medicine, when treating patients for tuberculosis infections stated that it was just as important to know what is going on in a man's head as in his chest (Leonard and Myint 2009). This statement is no longer eccentric. It is well established that events associated with elevated stress and an individual's negative attitude towards stress can increase their vulnerability to a number of infections and diseases, including upper respiratory tract infections and heart disease (Leonard and Myint 2009), whereas a positive attitude by a patient can prolong their life expectancy. Conversely, a disease can trigger changes in mood and the onset in depression. For example, a significant number of patients develop symptoms of a depressed mood six months prior to clinical signs of pancreatic cancer and approximately 10% of AIDS patients will show mood, behavioral, cognitive and memory changes before developing somatic signs of the illness (Leonard and Myint 2009). Probable candidates for producing many of the psychological changes associated with infections and diseases are pro-inflammatory cytokines that are produced by activated microglia and peripheral macrophages. For instance, hepatitis patients are often treated with the pro-inflammatory cytokine IFN-α and a significant number of these patients develop a depressed mood (Wichers et al. 2005).

A stroke is the disturbance of blood flow to the brain resulting in cell death and deficiencies in cell functioning (Sims and Muyderman 2010). There are two forms of stroke: ischemic stroke is the loss of blood supply to a region of the brain due to the blockage of a blood vessel, whereas hemorrhagic stroke is the accumulation of blood within or outside the brain due to a compromised or ruptured blood vessel. Approximately 87% of strokes are a result of ischemia (Donnan et al. 2008). Following ischemia, brain tissue is deprived of oxygen and glucose and may undergo permanent injury if deprived for more than three hours (Hacke et al. 2008). The brain is highly dependent on aerobic metabolism and therefore is especially susceptible to decreases in oxygen and glucose. As oxygen and glucose become depleted in a brain region, the production of mitochondrial energy compounds, such as ATP, decline (Saiki et al. 1997). Resulting ATP depletion leads to the malfunction of energy-dependent processes, such as the sodium-potassium pump that help maintain ion gradients across cell membranes. Glutamate transporters are highly dependent on the sodium ion gradient for the uptake of glutamate into a cell; however, disturbances in ion gradients can cause these transporters to reverse their uptake and release glutamate into the extracellular space (Tsuchiya et al. 1977). Finally, excess glutamate acts on glutamatergic receptors to stimulate an overbearing calcium influx into cells that activates enzymes to break down proteins necessary for cell survival. This is just one mechanistic outcome in ischemia, and many more factors are involved, including oxygen free radical production, lactic acid accumulation, and resulting damage to blood vessels and endothelium.

Depression is a common co-morbid disorder that accompanies chronic diseases such as Parkinson's and Alzheimer's disease and acute insults such as stroke (Even and Weintraub 2010; Raskin and Durst 2010; Weintraub et al. 2010; Arbus et al. 2011; El Husseini et al. 2012). Depression is one of the most common disorders appearing following a stroke, and is also a major factor limiting recovery and rehabilitation in stroke patients (Esparrago Llorca et al. 2012). In a nine-year follow-up study, Li et al. (2012) found that patients diagnosed with depression had higher rates of stroke and these rates were dependent on the severity of depression. The primary treatment for depression before or after a stroke is SSRI antidepressants. These antidepressants have been shown to alleviate the depressed state and sleep disturbances in patients diagnosed with post-stroke depression (Sunami et al. 2012). SSRI antidepressants have also been shown to reduce anhedonia and anxiety levels observed in rodents following a middle cerebral artery occlusion (MCAo), a model of ischemic stroke (Kronenberg et al. 2012). In addition to improving behavioral symptoms, SSRI antidepressants prevent the degeneration of specialized neurons following ischemic insult. For instance, chronic SSRI treatment prevents the degeneration of DA midbrain neurons and striatal atrophy following MCAo in rodents (Kronenberg et al. 2012). There is evidence that SSRI prevention of neuronal death is mediated by microglia. An *in vitro* study found that fluoxetine protects mesencephalic DA neurons from damage by mediating anti-inflammatory properties of microglia in co-culture; fluoxetine failed to protect DA neuronal cultures from an insult in the absence of microglia (Chung et al. 2011). In a similar co-culture paradigm, fluoxetine has been shown to protect DA neurons from neurotoxicity through inhibiting microglial NF-κB signaling (Zhang et al. 2012). In an *in*

vivo experiment, fluoxetine attenuated neuronal death in the CA1 and CA3 regions in the mouse hippocampus following kainic acid administration, a model used to mimic the effects of glutamate excitotoxicity (Jin et al. 2009); however, the experiment failed to distinguish if microglia mediated the effects of fluoxetine.

Reduced blood flow during ischemic stroke results in energy deprivation, metabolic injury, excitotoxicity, and microglial activation. There is much debate as to the beneficial or detrimental role of activated microglia after stroke. In support of detrimental role of activated microglia, knockout or pharmalogical inhibition of the cytokines TNF-α and IL-1β improves stroke outcome (Wang and Shuaib 2002). In addition, treatment with anti-inflammatory drugs such as minocycline and doxycycline promote tissue survival after stroke in rodents (Jantzie et al. 2005). Conversely, ablation of microglia with the drug ganciclovir exacerbates tissue damage after stroke, supporting a beneficial role for microglia (Lalancette-Hebert et al. 2007). Therefore, it is possible that moderate activation of microglia may remove toxins and debris from the injury site to promote neuroprotection; however, over-activation without intervention may cause toxicity additional to the initial insult.

1.8. THESIS OBJECTIVES

1.8.1. Antidepressant effects on the phenotype of activated microglia

There has been significant progress supporting the inflammatory hypothesis in depression. The principles behind previous hypotheses focusing on monoamines, HPA axis activity, and neurogenesis failed to sufficiently describe the mechanisms involved in

depression. A plethora of literature suggests that a dysfunction of inflammatory signaling may help explain the etiology of depression and the effectiveness of antidepressant drugs. Several studies have investigated the effects of antidepressants on the inflammatory profiles of activated and resting microglia. We can compare the effects of various classes of antidepressants from a range of studies conducted by various groups; however, we are the first to investigate the effects of three classes of antidepressants (MAOIs, TCAs, and SSRIs) on microglia all within one study. Our objective was to measure and compare the release of pro-inflammatory mediators (NO, TNF-α, and IL-1β) and anti-inflammatory mediators (BDNF, IL-4, and GDNF) from LPS-activated and resting microglia following antidepressant treatments. We have chosen to investigate two antidepressants from each class, and all have been previously shown to be effective in alleviating depressive symptoms in patients. We hypothesized that all of the antidepressant drugs under investigation in this study would alter the inflammatory and trophic profile of activated microglia.

1.8.2. Antidepressant effects on cortical neuronal viability after ischemic insult

As mentioned earlier, stroke and depression are strongly correlated with one another. There is a larger probability of episodes of ischemic stroke in depressed patients and depression is the most common comorbid disorder following stroke. Antidepressants are the drug of choice to treat post-stroke depression. After an ischemic insult, antidepressants can prevent neuronal death in dopaminergic and hippocampal brain regions, most likely by mediating the inflammatory profile of microglia. However, depending on the location of the stroke, other neuronal subtypes are affected. For

instance, ischemic insult in several regions of the brain affects cortical neurons. Cortical neurons are critical in mediating a number of factors including motor output, sensory input, cognition, and speech. However, in literature, there is a lack of studies investigating antidepressant effects on the survival and viability of cortical neurons after ischemic insult. Therefore, one of our objectives was to investigate if antidepressants can affect the neuronal viability of cortical neurons following ischemic insult. We also investigated if any resulting effects of the antidepressants on cortical neuronal viability are mediated through microglia. We hypothesized that all of the antidepressants we investigated would promote neuronal survival and that this survival is based on effects of the antidepressants on microglial function.

1.8.3. The microglial factors important in mediating cortical neuronal viability

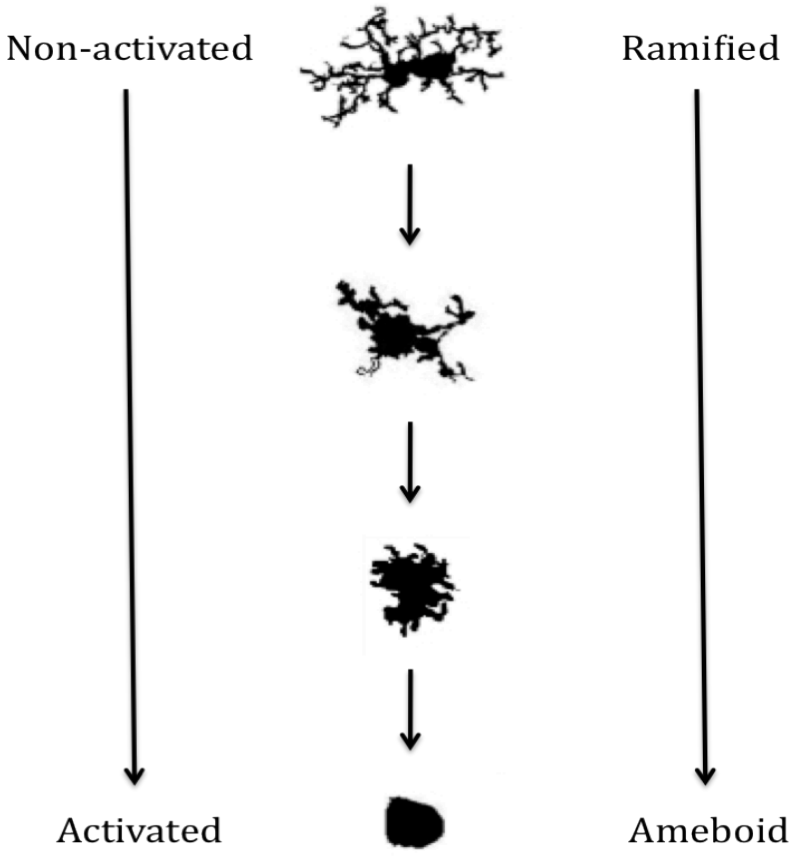
It is already established that antidepressants can alter the release profile of a number of mediators from microglia. However, it is unclear which microglial-derived mediators are the key contributors in mediating neuronal viability following antidepressant treatment. Microglial release of amino acids, primarily glutamate and D-serine, has been reported after activation (Wu et al. 2004; Jin et al. 2007), but the effects of antidepressants on this amino acid release have yet to be investigated. Levels of amino acids, primarily glutamate, are important determinants in the outcome of stroke, and previous studies in the literature report alterations in amino acid signaling following excessive inflammation. Our objective was to investigate antidepressant effects on amino acid release from LPS-activated and resting microglia. In addition, we determined the key contributors from microglia in mediating cortical neuronal viability following an ischemic insult through

rigorous antagonism experiments: Are the key contributors pro- or anti-inflammatory cytokines, trophic factors, or amino acids? Excessive microglial induced inflammation is typical after stroke and leads to further toxic effects on surrounding cortical neurons. Therefore, we hypothesized that antagonizing the toxic mediators from activated microglia will promote cortical neuron viability.

1.8.4. The intracellular mechanisms of antidepressant effects on microglia

There are numerous studies that investigate the internal signaling cascades in microglia following antidepressant treatment. However, these studies fail to compare effects of antidepressant drugs from different classes on the expression of signaling proteins within microglia. Our objective was to determine the mechanisms underlying the anti-inflammatory actions of various classes of antidepressants in microglia. We compared secondary messenger pathways within LPS-activated and resting microglia following antidepressant treatments by measuring the expression of two proteins linked to the TLR4 pathway, namely p38 MAP kinase and NF-κB. By measuring these proteins, we can get a better understanding about at what step in the signaling cascade antidepressants have their effects in microglia. We hypothesized that those antidepressants that alter the inflammatory profile of activated microglia will also alter the expression of p38 and NF-κB in activated microglia. An unexpected result following observations in fluoxetine-treated microglia led us to believe that fluoxetine induced microglial apoptosis. To further investigate this finding, we measured the expression of pro- and cleaved-forms of the apoptotic-linked proteins, namely caspase-3 and caspase-8, in microglia.

1.9. Figures



Modified from Karperien et al., 2013

Figure 1-1 – The morphology of non-activated and activated microglia. Microglia are dynamic cells able to change form in response to an activator. This change in microglial form involves a change from a highly branched (ramified) phenotype to one that lacks processes (amoeboid) in response to an activator.

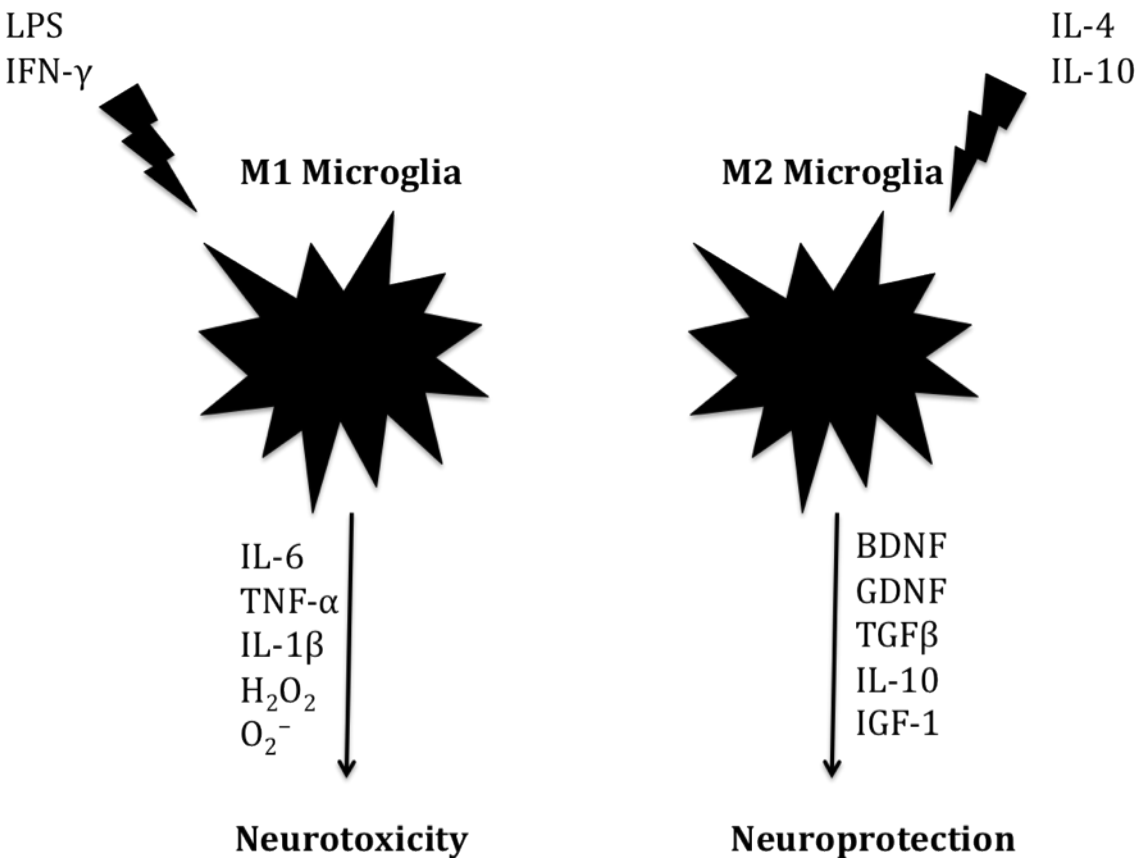


Figure 1-2 – The differences between M1 and M2 activated microglia. The M1 microglial phenotype releases a variety of pro-inflammatory factors upon activation and promotes neurotoxicity. The M2 microglial phenotype releases a variety of anti-inflammatory factors upon activation and promotes neuroprotection.

1.10. References

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CHAPTER 2

Antidepressant effects on the activated profile of microglia

2.1. Introduction

2.1.1. *In vitro* vs. *in vivo* experimentation

Most experimental protocols are one of two types, *in vitro* or *in vivo*. *In vivo* studies refer to the manipulation within whole, living organisms and are suited for observing the overall effects of an experiment on a living subject. A disadvantage with *in vivo* studies is it is difficult to isolate a certain cell or factor of interest after a manipulation. For instance, it is hard to pinpoint the source of cytokine elevations in depressed patients during clinical trials. Therefore, *in vitro* studies are used to isolate the factor of interest in a study. *In vitro* refers to an experiment performed outside a living organism in a controlled environment and allows for the study of cell-specific interactions. A disadvantage of *in vitro* studies is that they fail to reproduce the complete cellular environment of a living organism and certain cells such as microglia may not function identically as they would *in vivo*. Hurley et al. (1999) have shown that primary cultured microglia taken from postnatal day 4 supraventricular corpus callosum (SVCC) abundantly express mRNA transcripts for TNF-α, IL-1β, and macrophage colony-stimulating factor, but these transcripts are very low to undetectable in the SVCC *in vivo.* This difference may be explained with the medium utilized to grow primary cell cultures as the extracellular fluid composition may differ from organism to organism or between tissues and these differences are difficult to replicate *in vitro.* Consequently, it is important to use caution when interpreting *in vitro* findings in terms of the *in vivo* functions of microglia. The most reliable data stems from the combination of both *in vitro* and *in vivo* work. For example, Chung et al. (2011) showed that fluoxetine is neuroprotective of dopaminergic neurons under toxic conditions in neuronal-microglia

co-cultures *in vitro*, and then supported their findings with *in vivo* work as they found fewer degenerated dopaminergic neurons and significant motor recovery in fluoxetine-treated mice following toxic insult.

In the studies described in this thesis, I used *in vitro* methodology based on our interest in the interactions between two cell types within the brain, microglia and cortical neurons. In this chapter, the goal of the experiments described was to successfully culture and isolate microglia from the rat brain with minimal contamination from other cell types. Cells can be cultured as a primary cell culture or a cell line. A primary cell culture consists of cells cultured directly from a subject, whereas cell lines are cells that can indefinitely proliferate through various genetic modifications. The advantage of a cell line is that the cells have a very long lifespan and require the use of fewer animals; however, the downside is that genetic manipulation of cells may alter the response of cells to various stimuli. Therefore, primary cell cultures are preferred due to the lack of genetic manipulation, even though the cells may not survive as long as in a cell line.

2.1.2. Isolation of microglia in culture

In order to obtain an isolated microglial culture, the first step is to grow the cells as a mixed culture. A mixed culture yields a number of cell types found within the brain including microglia, astrocytes, oligodendrocytes, endothelial cells, ependymal cells, and possibly neurons. Once a mixed culture has reached confluence, microglia can be isolated from other contaminating cells primarily by three methods, namely shaking, density gradient centrifugation, and trypsinization. Microglial isolation by shaking

involves robust mechanical shaking of mixed cultures to detach microglia from the surface of a flask or plate. Microglia adhere less to the surface of a flask or plate compared to other cell types in mixed cultures. These floating cells can be cultured in a separate plate to yield isolated microglia. However, there are many disadvantages to the shaking method. For instance, the shaking procedure takes a lengthy time to obtain a good yield of microglial cells, approximately 2-3 days (Tamashiro et al. 2012). Even after the first round of shaking, other cell types can also detach and contaminate floating microglia, and another round of shaking is required to exclude these cells, thereby increasing the length of an experiment. In addition, the yield of microglia is very low compared to the trypsinization method (Saura et al. 2003). Primary microglia can be isolated through the process density gradient centrifugation of homogenized brain tissue where cell types are separated after a period of centrifugation based on their densities with respect to one another; however, this centrifugation, typically of 45 min in length, may cause cellular damage and activation (Tamashiro et al. 2012).

2.1.3. Isolation of microglia by trypsinization

Trypsinization is an isolation method where all mixed culture cells except microglia detach from the surface of a flask or plate, thereby yielding a pure culture of microglia. The dilution of trypsin, a protease, with calcium-rich medium helps detach cells from a plate surface without affecting the microglial attachment; however, if trypsin is diluted in calcium-free medium, all cells including microglia become detached and begin to float (Saura et al. 2003). One explanation behind these findings is that calcium may influence

the functioning of a particular integrin that is abundantly expressed on microglia and not the other cell types, but the exact role of calcium in isolation needs to be elucidated.

The advantages of trypsinization over shaking include the length of time required (30 minutes vs 3 days) and a higher yield of microglial cells. A disadvantage of trypsinization is that trypsin may alter the properties of microglia by activating the cells; however, following trypsinization, overnight incubation in trypsin-free medium reverts microglia back into the resting state, but it remains controversial if trypsin-induced activation influences the properties of microglial activation when exposed to additional stimuli. Nonetheless, trypsin-induced microglial activation should not be used as a deterrent from using trypsinization since mechanical shaking can also activate microglia (Tamashiro et al. 2012).

2.1.4. Visualization of cells with immuno-fluorescence

Immunuo-fluorescence can be used to characterize and compare mixed cultures to isolated microglial cultures and is defined by the visualization of a specific molecule or antigen with fluorescence-labeled compounds. Specific antibodies recognizing astrocytes, oligodendrocytes, and microglia are used to distinguish the various cell types in mixed cultures and to verify the purity of isolated cultures. There are many antibodies that recognize cell-specific markers in differing cell types, and examples are given in Table 1.

TABLE 1

Marker	Label for	References
Iba1 (ionized calcium binding adaptor molecule 1)	Microglia	(Ito et al. 1998; Kuzumaki et al. 2010)
CD68 (ED-1 monoclonal)	Activated microglia	(Engelsberg et al. 2004; Ramanan et al. 2009)
CD11b (OX-42 monoclonal)	Microglia	(Terayama et al. 2008; Tozaki-Saitoh et al. 2008)
GFAP (glial fibrillary acidic protein)	Mature astrocytes	(Kuzumaki et al. 2010; Ahlemeyer et al. 2012)
CNPase (2',3'-Cyclic-nucleotide 3'-phosphodiesterase)	Embryonic, young, mature oligodendrocytes, schwann cells	(McEwan 1996; Kerr et al. 2010)
MBP (Myelin basic protein)	Mature oligodendrocytes, schwann cells	(Fragoso et al. 2007; Schmidt et al. 2010)
MAG (Myelin associated glycoprotein)	Embryonic, young, mature oligodendrocytes	(Keita et al. 2002; Fragoso et al. 2007)
DAPI (4',6-diamidino-2-phenylindole)	Cellular nuclei	(Agrawal and Godar 2012; Lee et al. 2012)

For our purposes, antibodies recognizing Iba1, GFAP, CNPase, and MAP-2 along with the nuclear marker, DAPI, are used to identify microglia, astrocytes, oligodendrocytes, neurons, and cellular nuclei, respectively, in both mixed cultures and isolated microglia cultures.

2.1.5. LPS activation through TLR4 signaling

The purpose of the studies described in this chapter was to determine the effect of antidepressants on the phenotypes of activated and resting microglia; therefore, we required a microglial activator. Yao et al. (2013) demonstrated that TLR4 mediates the microglial activation and production of inflammatory mediators following hypoxia. The

group found that TLR4 immunofluorescence, TLR4 protein expression, and the expression of pro-inflammatory factors (NO and TNF-α) were enhanced following hypoxia in rat neonatal microglia both *in vivo* and in primary cell cultures. siRNA knockdown of TLR4 reduced this hypoxia-induced increase of these pro-inflammatory factors in cultured cells and inhibition of TLR4 *in vivo* decreased the immunoexpression of these factors. Therefore, we decided to use LPS, a potent activator of microglia that induces the release of a wide range of inflammatory mediators often seen in stroke (Vartanian et al. 2011; Yousuf et al. 2012). LPS is an endotoxin derived from the outer membrane of gram-negative bacteria and binds to TLR4 (**Figure 2-1**). The TLR4 consists of an IL-1 receptor-like domain called the toll/IL-1 receptor (TIR) domain. The TIR domain can mediate two pathways, the MyD88-dependent and MyD88-independent pathways (Akira et al. 2001). The MyD88-dependent pathway involves the recruitment of IL-1 receptor-associated kinase (IRAK) to TLR4 by MyD88 upon stimulation with LPS. IRAK is activated by phosphorylation and associated with TNF receptor-associated factor 6 (TRAF6) that leads to the activation of two separate signaling pathways, namely NF-κB and MAP kinase, and these factors go on to transcribe many inflammatory cytokines (Lu et al. 2008). The MyD88-independent pathway transcribes primarily IFN-β through the activation of TIR-domain-containing adapter-inducing interferon-β (TRIF) and interferon regulatory factor 3 (IRF-3).

2.1.6. Chapter objectives

Antidepressant effects on microglial activation have been studied extensively; however, no study has compared these effects between different classes of antidepressants. Here

we looked at antidepressants in the TCA, MAOI, and SSRI classes and their effects on microglial activation at various doses. The microglial release factors that we examined were NO, TNF-α, and IL-1β following LPS and antidepressant treatment.

2.2. Materials and methods

2.2.1. Primary mixed glial cultures

Mixed glia were prepared from whole brains of postnatal day 1 Sprague-Dawley rats. Meninges were removed and brains were chopped and dissociated for 25 min by enzymatic digestion with 0.25% trypsin-EDTA, followed by mechanical triturating in Dulbecco's Modified Eagle Medium (DMEM/F-12, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 2% penicillin/streptomycin (P/S, Invitrogen). Mixed glia were plated on poly-L-lysine-coated (2 μg/ml, Sigma) 24-well plates at a density of 1 x 10^5 cells per well in DMEM/F-12 medium. Density was determined using trypan blue staining using a hemocytometer. Mixed glia were incubated at 37°C with 5% CO_2 and were fed every 3-4 days *in vitro* (DIV) with fresh medium. Mixed glia were cultured for 21 days to confluence.

2.2.2. Primary microglia culture

After 21 DIV, microglia were isolated from mixed glial cultures via trypsinization (Saura et al. 2003). Medium was removed from mixed cultures and the wells were washed with serum-free DMEM/F-12. After aspiration of washing medium, trypsin diluted in DMEM/F-12 (1:3 dilution) was added to the wells for approximately 30 min. The resulting detached cells and debris were aspirated and fresh medium supplemented with

10% FBS and 2% P/S were added to the wells. Plates were then incubated at 37°C with 5% CO_2 for 10 min to inactivate trypsin. Following inactivation, medium was aspirated and serum-free medium supplemented with 2% P/S was added to the wells. Cultures were incubated overnight at 37°C with 5% CO_2 and used for experiments the following day.

2.2.3. Immunocytochemistry

Mixed glia or isolated microglia were fixed with 10% formalin for 10 min. After 3 washes with phosphate-buffered saline (PBS), the cells were incubated with a blocking solution [10% normal horse serum (Gibco) in PBS with 0.1% Triton X-100] for 1hr. After washing, cells were incubated with primary antibodies recognizing Iba1 (1:1000, Wako), GFAP (1:1000, Dako), CNPase (1:1000, Sigma), and MAP-2 (1:500, Sigma) (all diluted in PBS with 1% normal horse serum) overnight at 4°C. After 3 additional washes with PBS, wells were incubated with the relevant fluorescent-conjugated secondary antibodies (1:200, Invitrogen) diluted in PBS for 1hr. After a final wash, wells were cover-slipped using aqueous mounting medium.

In addition, isolated microglia were co-labeled with Iba1 and DAPI mounting medium to rule out contamination by other cell types. Lieca imaging software coupled to an inverted fluorescent microscope (model DM16000B) was used to capture images of mixed and isolated cultures. Nine images were captured per well for each filter. Merged images were created by Image-J software. Cell counting of DAPI- and Iba1-labeled cells was performed with Metamorph™ software and verified by human counts.

To compare microglial morphologies before and after LPS treatments we used 3,3'-diaminobenzindine (DAB) visualization. The procedure is similar to immuno-fluorescence up to the primary antibody step. Following overnight incubation with the rabbit anti-Iba1 primary antibody, wells were incubated with anti-rabbit biotinylated secondary antibody (Wako) for 1 hr. Wells were then incubated with an ABC complex (1:200, ThermoScientific) for 1hr. Brown DAB tablets (Sigma) were dissolved in 8 ml of PBS and 3% hydrogen peroxide was added to the mixture (1:100). DAB was quickly added to the wells for approximately 2 min and the wells were visualized under a phase contrast microscope (Hund Wilovert 30). Wells that were not incubated with the anti-Iba1 primary but incubated with the secondary antibody and DAB were used as negative controls. Pictures were taken with a SLR Nikon D90 camera.

2.2.4. Antidepressants/chemicals

Three classes of antidepressants were assessed, namely TCAs (imipramine and clomipramine, Sigma), MAOIs (phenelzine and tranylcypromine, Sigma) and SSRIs (fluoxetine, Eli Lilly, and citalopram, H-Lundbeck). As a microglial activator, LPS (Sigma) was chosen.

2.2.5. Drug treatments

LPS was added to microglia at the following concentrations: 0 μg/ml, 0.1 μg/ml, 0.5 μg/ml, 1 μg/ml, and 2 μg/ml. We found that LPS at 1 μg/ml significantly increased the production of NO compared to the other concentrations tested, excluding 2 μg/ml with

which there was no significant difference. The LPS concentration of 1 μg/ml was used in further experiments. The following antidepressant doses were tested on activated and resting microglia: 0 μM, 5 μM, 10 μM, 20 μM, 30 μM, and 40 μM, where NO, TNF-α, and IL-1β release were measured in the medium. Antidepressants were added to microglia 30 min before LPS activation and the cultures were left to incubate for 24 hrs. Microglial cultures were randomly assigned to one of the following four conditions: Control (PBS only), LPS-treated, antidepressant-treated, and LPS + antidepressant-treated.

2.2.6. Nitrite assay and protein assay

Nitrite levels, an indirect measure of NO, were determined by the Griess Reaction as described in previous reports (Green et al. 1982; Lai and Todd 2008). Briefly, 100μl of differentially treated medium were incubated with 50μl of 1% sulfanilamide in 3N HCl and 50μl of 0.1% naphthylethylenediamine dihydrochloride (NED) in distilled water, in 96 well plates. Absorbance (540nm) was read on a plate reader (Powerwave X, Bio-Tek). Values were standardized to the absorbance levels of nitrite standard obtained from Promega.

For protein levels, medium was removed and wells were washed 3 times with PBS. Lysis buffer (0.8% Triton X-100 and 0.2% SDS in Tris-EDTA-EGTA-NaCl [TEEN] buffer) were added to the wells. The wells were placed on a shaker at 4°C for 20-30 min. A solution (190μl) consisting of copper (II) sulfate solution diluted in bicinchoninic acid (1:50; Sigma) was added to 10μl of cell lysates and the samples were left to incubate at

37°C for 30 min in 96 well plates. Absorbance (562nm) was read on a plate reader. Final values of nitrite were calculated after normalizing values to protein.

2.2.7. Enzyme-linked immunosorbant assay (ELISA)

TNF-α and IL-1β quantification was performed using ELISA kits (R & D systems). The assays were conducted based on the protocols supplied by the manufacturer. Capture antibodies (4 μg/ml and 0.8 μg/ml for TNF-α and IL-1β, respectively) were added to 96 well plates and left overnight. The wells were washed and blocked with 1% bovine albumin (BSA) in PBS for 1 hr. Samples and standards (100 μl) were added to the wells and left to incubate overnight at 4°C. After washing wells with PBS, 100 μl of detection antibody (225 ng/ml and 350 ng/ml for TNF-α and IL-1β, respectively) were added to the wells and the samples left to incubate for 2 hrs at room temperature. After washing wells with PBS, 100 μl of HRP-conjugated streptavidin secondary antibody, diluted 1:200 from the stock, were added to the wells and left to incubate for 20 min at room temperature. After a final wash, a mixture of 30% hydrogen peroxide diluted in a solution of tetramethylbenzidine (TMB, Sigma) (1:1000) was added to the wells and the samples were left for approximately 20 min. The TMB solution was prepared by dissolving 8.2 g sodium acetate and 3.6 g citric acid in 600 mL of double distilled water. In a separate beaker, 270 mg TMB was dissolved in 400 mL methanol, which required heating to dissolve. The two solutions were then mixed together to form 1 L and stored at -20°C until needed for experimental use. Prior to use, TMB solution was reheated, dissolved, and filtered. The reaction was stopped by adding 1.8 N sulfuric acid to the wells and the absorbance was read at 450 nm using a plate reader. Plates were also read at 570 nm to

account for background and these values were subtracted from the 450 nm readings. Values were normalized to protein levels.

2.2.8. Statistical Analysis

Significance levels for data sets were calculated using a two-tailed t-test (significance designated with *) or a two way ANOVA followed by the Bonferroni post hoc test (designated with #). t-Tests were used to compare two data sets involving the manipulation of only one variable. Two way ANOVAs were used to compare two data sets involving manipulations of two or more variables. Both designations * and # denote $p < 0.05$. Refer to figure legends for total 'n' values for specific experiments. For all NO and ELISA experiments, each 'n' represents one set of cultures with a replicate of three wells per condition.

2.3. Results

2.3.1. Trypsinization yields pure isolated microglia from mixed glial cultures

The first step was to obtain pure microglial cultures and eliminate the contamination of other cell types. The most abundant cell types in mixed glial cultures are astrocytes, microglia, and oligodendrocytes. Therefore, we labeled for these various cell types to first verify the absence of contaminating cells in isolated cultures and second, used mixed glial cultures as positive controls for primary antibody labeling.

Mixed glial cultures showed labeling of CNPase-positive oligodendrocytes (**Figure 2-2 A and B**), GFAP-positive astrocytic processes (**Figure 2-3 A** and **B**), and Iba1-positive

microglia (**Figure 2-2 A** and **B, Figure 2-3 A** and **B, Figure 2-4 A** and **B**). Merged images of Iba1-positive microglia with either CNPase-positive oligodendrocytes or GFAP-postive astrocytes displayed no co-labeled cells, verifying that the primary antibodies label distinct cell types. The most abundant cells in mixed cultures of the three labeled were astrocytes, followed by microglia, and finally oligodendroctyes. In addition, we labeled for neurons using microtubule-associated protein-2 (MAP-2) in mixed glia cultures. By three weeks of culture, glial cells normally proliferate and occupy the surface area of a well, thereby minimizing the growth of non-mitotic cells such as neurons; therefore we expected to observe minimal expression of MAP-2 in mixed cultures. As expected, mixed cultures showed no MAP2-positive neurons (**Figure 2-4 A** and **B**).

Following trypsinization, isolated cultures no longer displayed the labeling of CNPase-positive oligodendrocytes (**Figure 2-2 C** and **D**) and GFAP-positive astrocytic processes (**Figure 2-3 C** and **D**), but displayed Iba1-positive microglia (**Figure 2-2 C** and **D, Figure 2-3 C** and **D, Figure 2-4 C** and **D**). As expected, isolated cultures showed no MAP2-positive neurons (**Figure 2-4 C** and **D**). In addition, isolated cultures showed a lower yield in the number of microglia compared to mixed cultures, suggesting that some microglia also lift from the surface of a well during or following trypsinization.

To verify that other cell types such as endothelial and ependymal cells did not contaminate the isolated microglial cultures, we co-labeled the cells with Iba1 and DAPI, a fluorescent stain that labels the nuclei of all cells. Theoretically, in an isolated pure

microglial culture all DAPI-stained cells will co-label with Iba1. We found that 99.7% of the isolated cells co-labeled for DAPI and Iba1 (**Figure 2-5 A** and **B, Figure 2-5 D**). As a negative control, cells were incubated with the suitable fluorescent-conjugated secondary antibody without the incubation of anti-Iba1 primary. This step rules out any non-specific binding of the secondary antibody in the isolated culture. We found no labeling with the fluorescent secondary used against Iba1 in the negative controls, but it was evident in cells stained with DAPI, confirming the presence of cells (**Figure 2-5 C**). Therefore, we were confident in the purity of the isolated microglial cultures following the trypsinization of mixed glial cultures and used these cultures in further experiments in this thesis.

2.3.2. LPS activates microglia in a concentration-dependent manner

We treated isolated microglia with different concentrations of LPS (0 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml, 1 μg/ml, and 2 μg/ml) to determine the concentration-dependent effects of LPS on microglial activation. We measured the release of NO and TNF-α from microglia to determine the strength of activation and found that all the LPS concentrations tested significantly increased the microglial release of NO and TNF-α compared to non-treated cells (**Figure 2-6 A** and **B**). LPS at a concentration of 1 μg/ml significantly increased NO release from microglia compared to 0.1 μg/ml of LPS, whereas the concentration of 2 μg/ml showed an increase compared to 0.1 μg/ml but this did not reach significance (**Figure 2-6 A**). There were no other significant concentration-dependent effects of LPS on microglial NO release. In addition, there was no further effect of LPS on TNF-α release from microglia above 0.1 μg/ml (**Figure 2-6 B**).

To study microglial morphology following LPS treatments, we visualized Iba1-positive microglia with DAB. We found that the morphology of non-treated Iba1-positive microglia was ramified, characterized by the elongation of cells with numerous branches. Following the activation of microglia with 0.1, 0.2, or 0.5 μg/ml of LPS, many cells remained elongated, but there were a greater number of cells, perhaps through proliferation, and a greater number of spherical cells compared to non-treated microglia (**Figure 2-6 C**). Activating microglia with 1 or 2 μg/ml of LPS clearly shows a greater number of spherical cells in addition to a greater number of total cells compared to non-treated microglia and cells treated with the other LPS concentrations (**Figure 2-6 C)**. Because 1 μg/ml of LPS increases the microglial release of NO and TNF-α and clearly changes the morphological properties of resting microglia, we decided to use this concentration as the activator of microglia in further experiments in this thesis.

2.3.3. Antidepressants attenuate the release of NO, TNF-α, and IL-1β from LPS-activated microglia

Our next step was to investigate if antidepressants attenuate the release of pro-inflammatory factors from LPS-activated microglia and if this effect is dose-dependent. Microglia were treated with various doses of antidepressants for 30 min prior to LPS activation (1 μg/ml) and the factors were measured 24 hrs later. Antidepressants had no effect on the release of NO, TNF-α, or IL-1β from non-activated microglia (**Figure 2-7, Figure 2-8, Figure 2-9**).

When microglia were treated with the TCAs (imipramine and clomipramine) prior to LPS activation, we found that all the doses investigated, excluding clomipramine at 5 μM, significantly attenuated NO and IL-1β release from activated microglia (**Figure 2-7, Figure 2-9**). Imipramine significantly attenuated the release of TNF-α from activated microglia at all doses investigated, excluding the dose at 5 μM, whereas clomipramine had no effect on the release of TNF-α from activated microglia at any dose (**Figure 2-8**). The release of NO, TNF-α, and IL-1β from TCA-treated activated microglia remained significantly greater compared to TCA-treated non-activated controls.

When microglia were treated with the MAOIs (phenezline and tranylcypromine) prior to LPS activation, we found that all the doses investigated, excluding tranylcypromine at 5 μM, significantly attenuated NO release from activated microglia (**Figure 2-7**). Phenelzine failed to decrease TNF-α release from activated microglia at any dose, whereas TCP decreased this TNF-α release at all doses tested (**Figure 2-8**). All doses, excluding the doses of 5 μM phenelzine and tranylcypromine, decreased the IL-1β release from activated microglia (**Figure 2-9**).

When microglia were treated with the SSRIs (fluoxetine and citalopram) prior to LPS activation, we found that all the doses investigated significantly attenuated the NO and TNF-α release from activated microglia (**Figure 2-7, Figure 2-8**). All investigated doses, excluding the dose of 5 μM citalopram, decreased the IL-1β release from activated microglia (**Figure 2-9**).

The lowest dose for most of the antidepressants that was consistently effective in attenuating the release of pro-inflammatory factors from activated microglia was 10 μM. Phenelzine and clomipramine were the only antidepressants that failed to decrease TNF-α release from activated microglia at this dosage. However, this finding is not concentration-dependent since higher doses of these antidepressants (20 μM, 30 μM, and 40 μM) also failed to decrease this release in TNF-α. The other antidepressants showed decreases in NO, TNF-α, and IL-1β release at 10 μM and higher doses did not significantly alter this decline. Therefore, in further experiments in this thesis, we used the dose of 10 μM for all the antidepressants tested.

2.4. Summary

This chapter accomplished a number of goals that lead to further experiments in this thesis. First, we successfully obtained a pure culture of microglia after the trypsinization method for isolation from mixed glial cultures. The immunofluorescent images showed minimal contamination by other cell types (astrocytes, oligodendrocytes, and neurons) in isolated microglial cultures. In addition, the majority of DAPI-labeled cells (label of all cell nuclei) also co-labeled with Iba1 (a microglial marker), thus supporting the purity of the isolated microglial cultures.

The next goal was to select a concentration of LPS to activate cultured microglia. We found that 1 μg/ml was the optimal concentration of activation based on the comparisons in the release of pro-inflammatory factors from microglia (NO and TNF-α) and the microglial morphologies between different concentrations of LPS-activation. The LPS

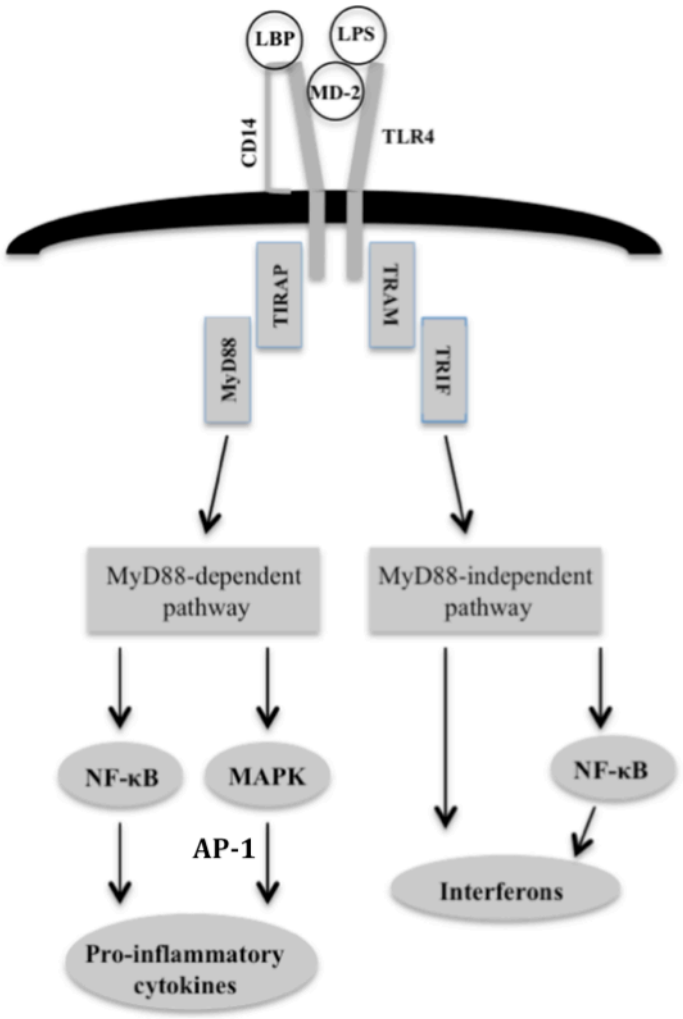
administration of 1 μg/ml showed the greatest NO release from microglia and morphological change compared to lower concentrations of LPS, whereas 2 μg/ml of LPS did not show any differences compared to the 1 μg/ml concentration. The release of TNF-α was not significantly different between any LPS concentrations tested. The morphology of microglia following 1 μg/ml showed a greater number of spherical cells (a mark of activation) and fewer elongated cells compared to the lower concentrations of LPS. Therefore, we were confident in utilizing a concentration of 1 μg/ml as a potent activator of LPS for further studies in this thesis.

Next, we investigated the effects of antidepressants on the release profiles of pro-inflammatory cytokines following the activation of microglia and compared antidepressants from three different classes (TCAs, MAOIs, and SSRIs). We also performed a dose-response study to see if antidepressant effects on this release were dose-dependent. We found that the majority of the antidepressants attenuated the release of the pro-inflammatory factors (NO, TNF-α, and IL-1β) at the doses 10 μM or higher. Higher doses of the antidepressants did not significantly attenuate the release in these factors compared to the dose of 10 μM. Phenelzine and clomipramine are two antidepressants that did not attenuate the release of TNF-α from activated microglia at any dose tested. Therefore, we selected the dose of 10 μM for all the antidepressants tested for further studies in this thesis.

The next chapter investigates the effects of antidepressants on cortical neuronal viability following an injury in a specialized co-culture setup containing both microglia and

cortical neurons. We determined if antidepressants attenuate the loss in neuronal viability in a co-culture with activated microglia and if this attenuation is an action directly on neurons or indirectly through effects on microglial activation. Based on the findings in this chapter, microglia were treated with 10 μM of antidepressants and activated with 1 μg/ml LPS for all future studies reported in this thesis.

2.5 Figures



Modified from Lu et al., 2008

Figure 2-1 - Overview of LPS/TLR4 signaling. LPS/TLR4 signaling can be separated into MyD88-dependent and MyD88-independent pathways, which mediate the activation of pro-inflammatory cytokine and Type I interferon genes. LBP – lipopolysaccharide binding protein; AP-1 – activator protein 1 transcription factor; TIRAP - toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TRIF - TIR-domain-containing adapter-inducing interferon-β; TRAM - TRIF-related adaptor molecule, MyD88 - Myeloid differentiation primary response gene 88; MAPK - Mitogen-activated protein kinases; NF-κB - nuclear factor kappa B.

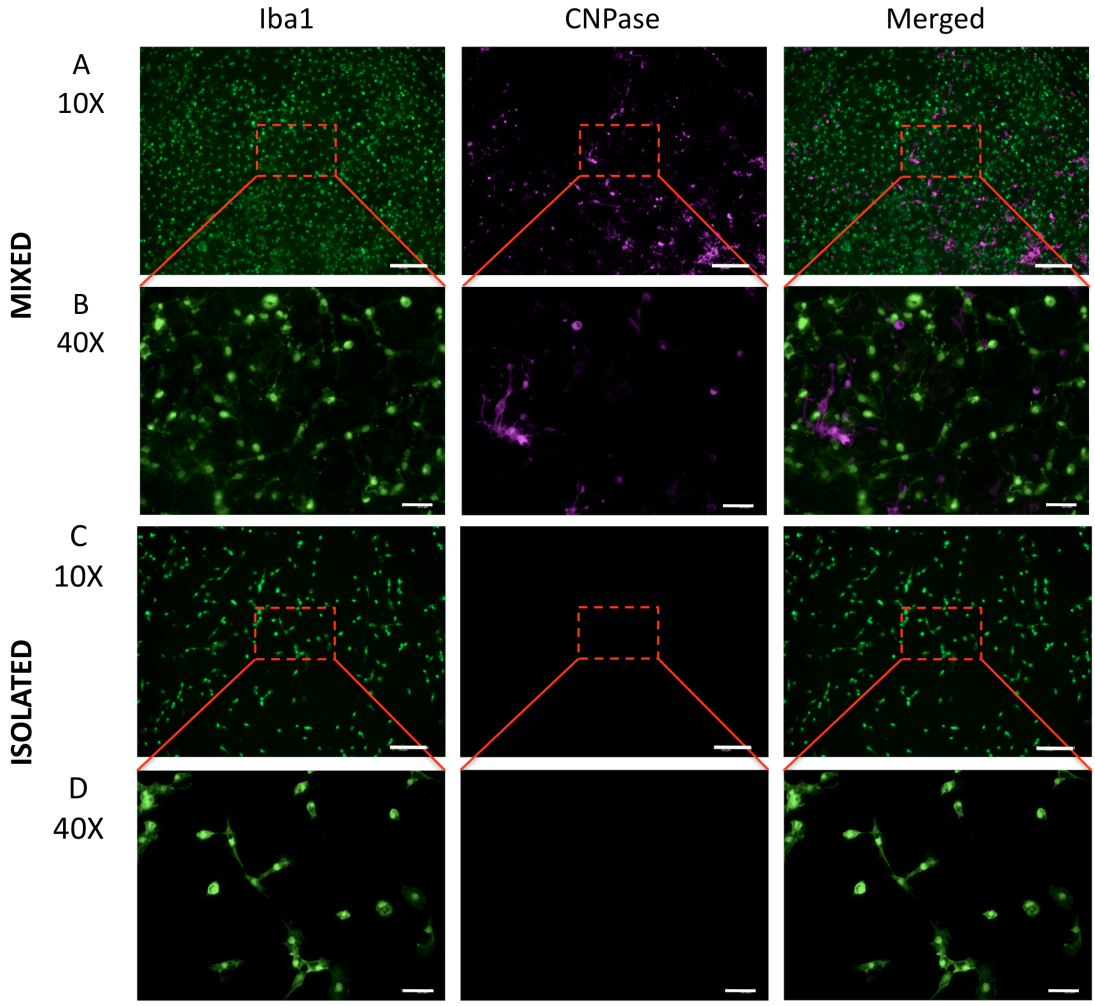


Figure 2-2 – Comparison between mixed glia and isolated microglia cultures for oligodendrocyte contamination. Mixed cultures showed presence of oligodendrocytes, whereas isolated microglial cultures showed no labeling for oligodendrocytes: **A.** Photomicrographs displaying Iba1 (microglia), CNPase (oligodendrocytes), and merged images from mixed cultures (scale bar = 150 μM, 10X objective). **B.** Higher magnification images of mixed cultures from A (scale bar = 30 μM, 40X objective). **C.** Photomicrographs displaying Iba1, CNPase, and merged images from isolated cultures (scale bar = 150 μM, 10X objective). **D.** Higher magnification images of isolated cultures from A (scale bar = 30 μM, 40X objective). Each image is a representative of one photomicrograph of 9 taken within each well. For each condition, 6 different wells across at least 3 separate animal preparations were assessed.

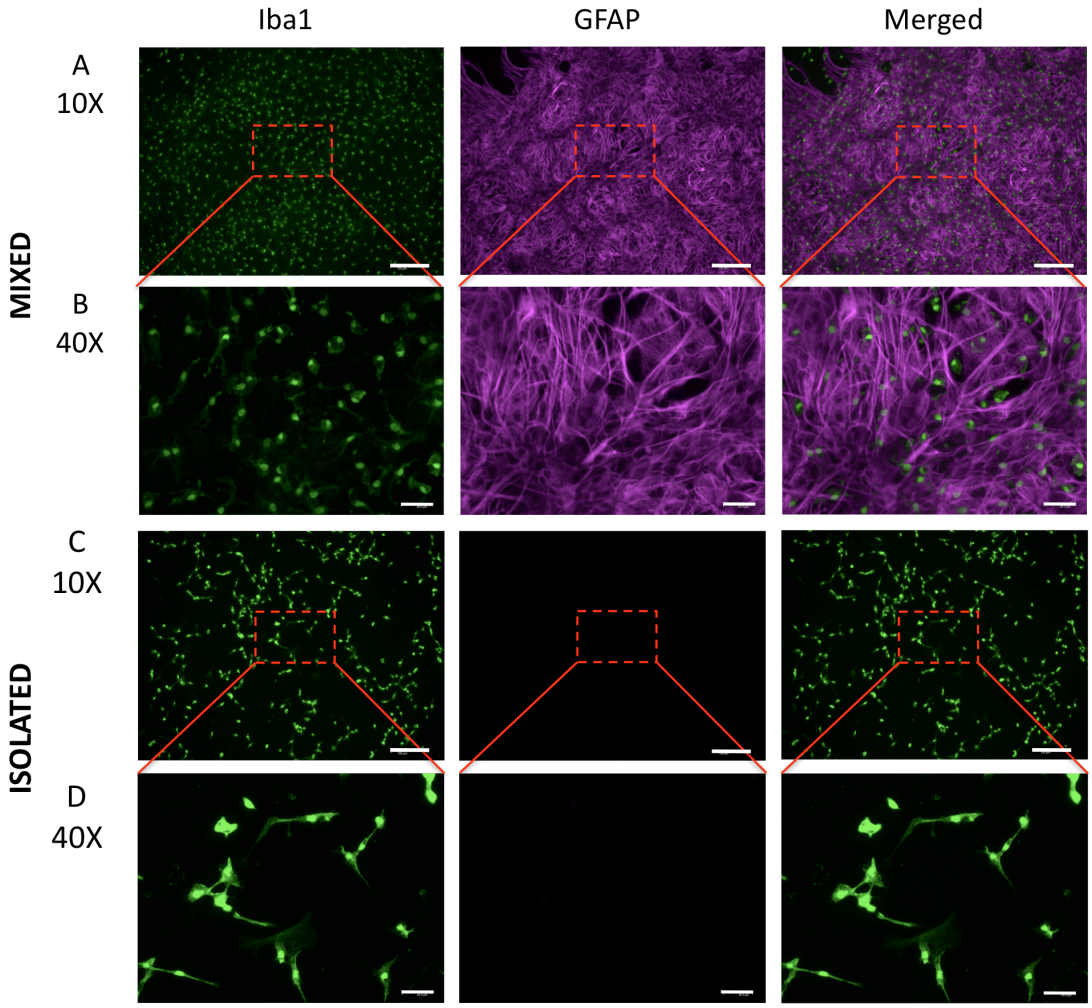


Figure 2-3 – Comparison between mixed glia and isolated microglia cultures for astrocyte contamination. Mixed cultures showed presence of astrocytes, whereas isolated microglial cultures showed no labeling for astrocytes: **A.** Photomicrographs displaying Iba1 (microglia), GFAP (astrocytes), and merged images from mixed cultures (scale bar = 150 μM, 10X objective). **B.** Higher magnification images of mixed cultures from A (scale bar = 30 μM, 40X objective). **C.** Photomicrographs displaying Iba1, GFAP, and merged images from isolated cultures (scale bar = 150 μM, 10X objective). **D.** Higher magnification images of isolated cultures from A (scale bar = 30 μM, 40X objective). Each image is a representative of one photomicrograph of 9 taken within each well. For each condition, 6 different wells across at least 3 separate animal preparations were assessed.

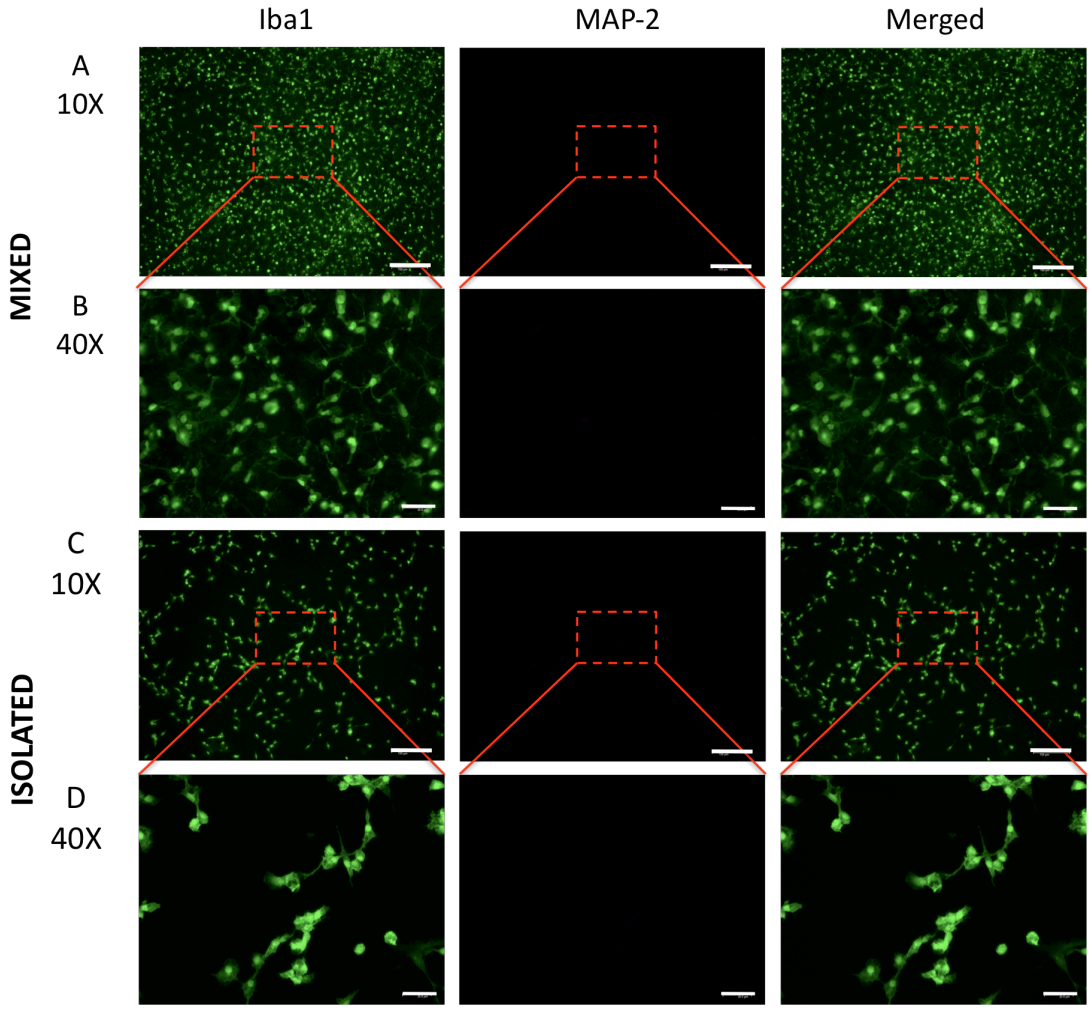


Figure 2-4 – Comparison between mixed glia and isolated microglia cultures for neuron contamination. Mixed cultures or isolated microglial cultures show no labeling of neurons: **A.** Photomicrographs displaying Iba1 (microglia), MAP-2 (neurons), and merged images from mixed cultures (scale bar = 150 μM, 10X objective). **B.** Higher magnification images of mixed cultures from A (scale bar = 30 μM, 40X objective). **C.** Photomicrographs displaying Iba1, MAP-2, and merged images from isolated cultures (scale bar = 150 μM, 10X objective). **D.** Higher magnification images of isolated cultures from A (scale bar = 30 μM, 40X objective). Each image is a representative of one photomicrograph of 9 taken within each well. For each condition, 6 different wells across at least 3 separate animal preparations were assessed.

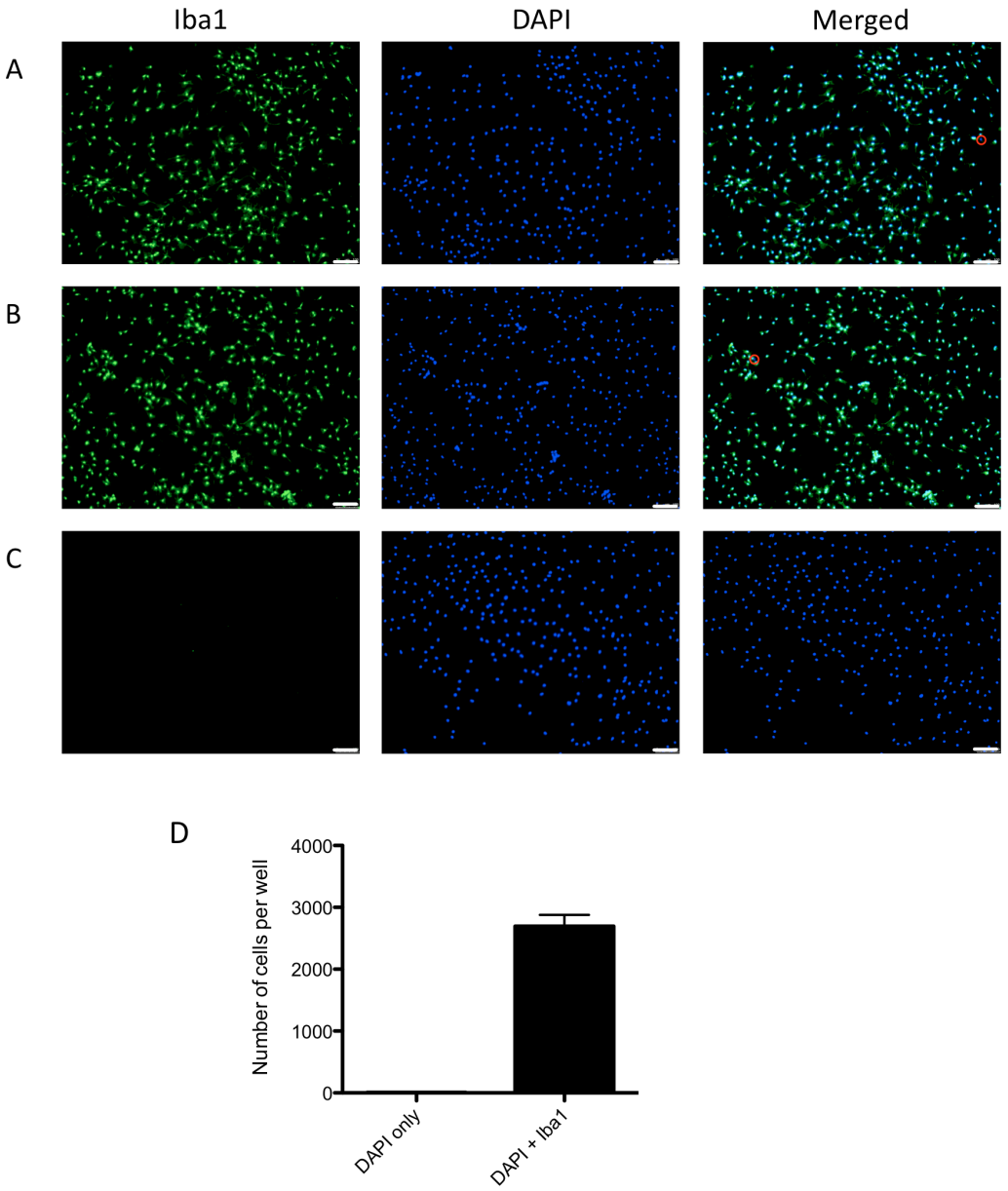


Figure 2-5 – Co-labeling isolated microglia with the nuclear stain DAPI. Iba1 (microglia) and DAPI co-label 99.7% in isolated cultures. **A and B.** Photomicrographs displaying Iba1, DAPI, and merged images from different fields of an isolated culture. Red circles are examples of cells stained for DAPI but negative for IBA1. **C.** Negatives displaying the lack of nonspecific binding of the secondary antibody in the absence of the Iba1 primary antibody. **D.** Cell counts of co-labeled cells and those only stained with DAPI (each image is a representative of one photomicrograph of 9 taken within each well. For each condition, 6 different wells across at least 3 separate animal preparations were assessed). Scale bar = 100 μM, 10X objective.

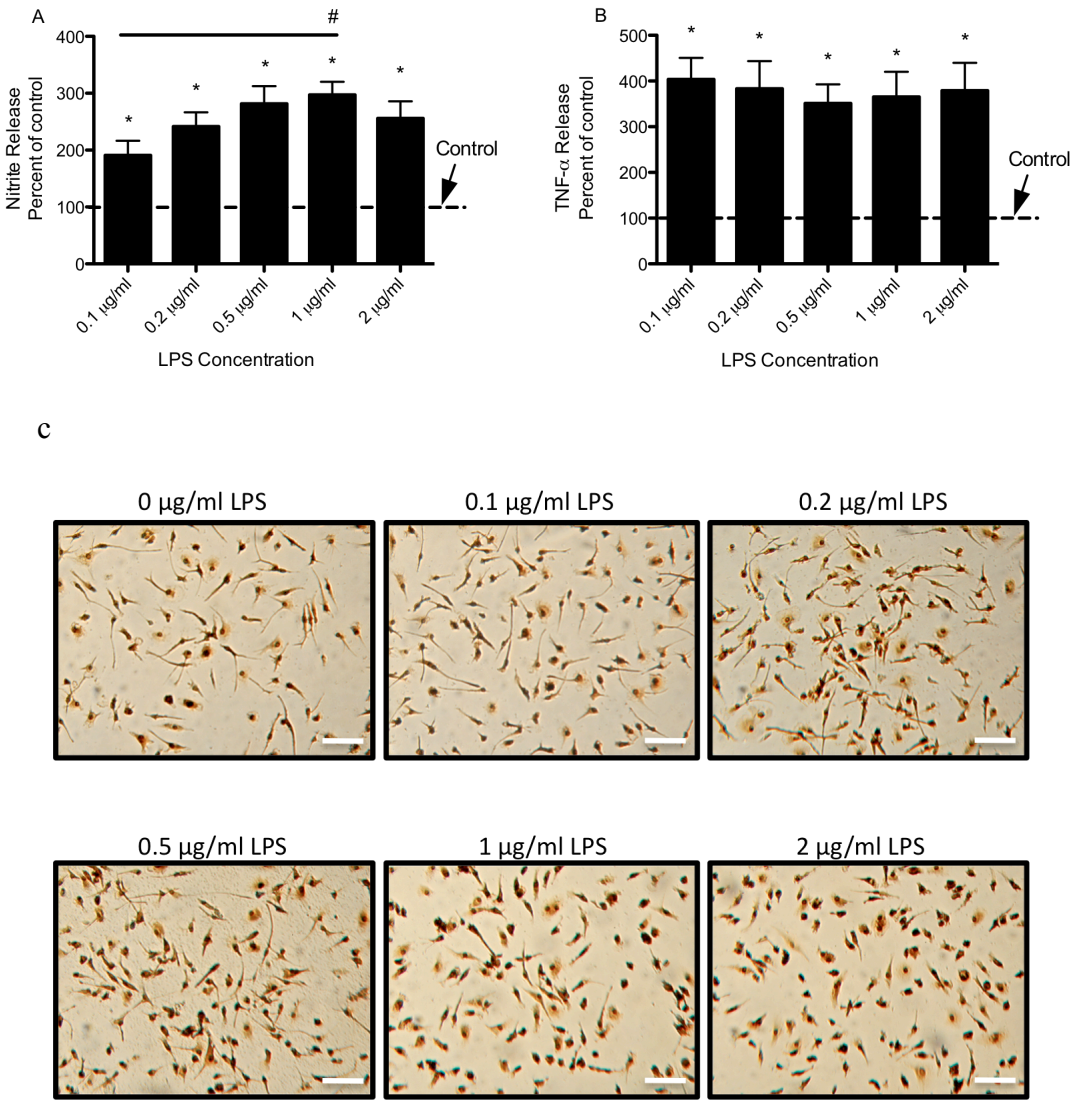


Figure 2-6 – LPS concentration effects on microglial release of NO and TNF-α and morphology. **A.** All LPS concentrations tested significantly induce microglial NO release. LPS at 1 µg/ml significantly induced greater NO release compared to 0.1 µg/ml; n = 8. **B.** All LPS concentrations tested significantly induce microglial TNF-α release. The concentration of LPS had no effect on TNF-α release; n = 8. **C.** DAB visualization of Iba1-postive microglia following LPS activation at various concentrations. The morphology of microglia changes from highly branched to spherical as LPS concentration increases; each image is a representative of one photomicrograph of 9 taken within each well. For each condition, 3 different wells across 2 separate animal preparations were assessed. Scale bar = 100 µM, 10X objective. Statistical significance, * < 0.05 (compared to control), #<0.05 (comparing LPS treament groups).

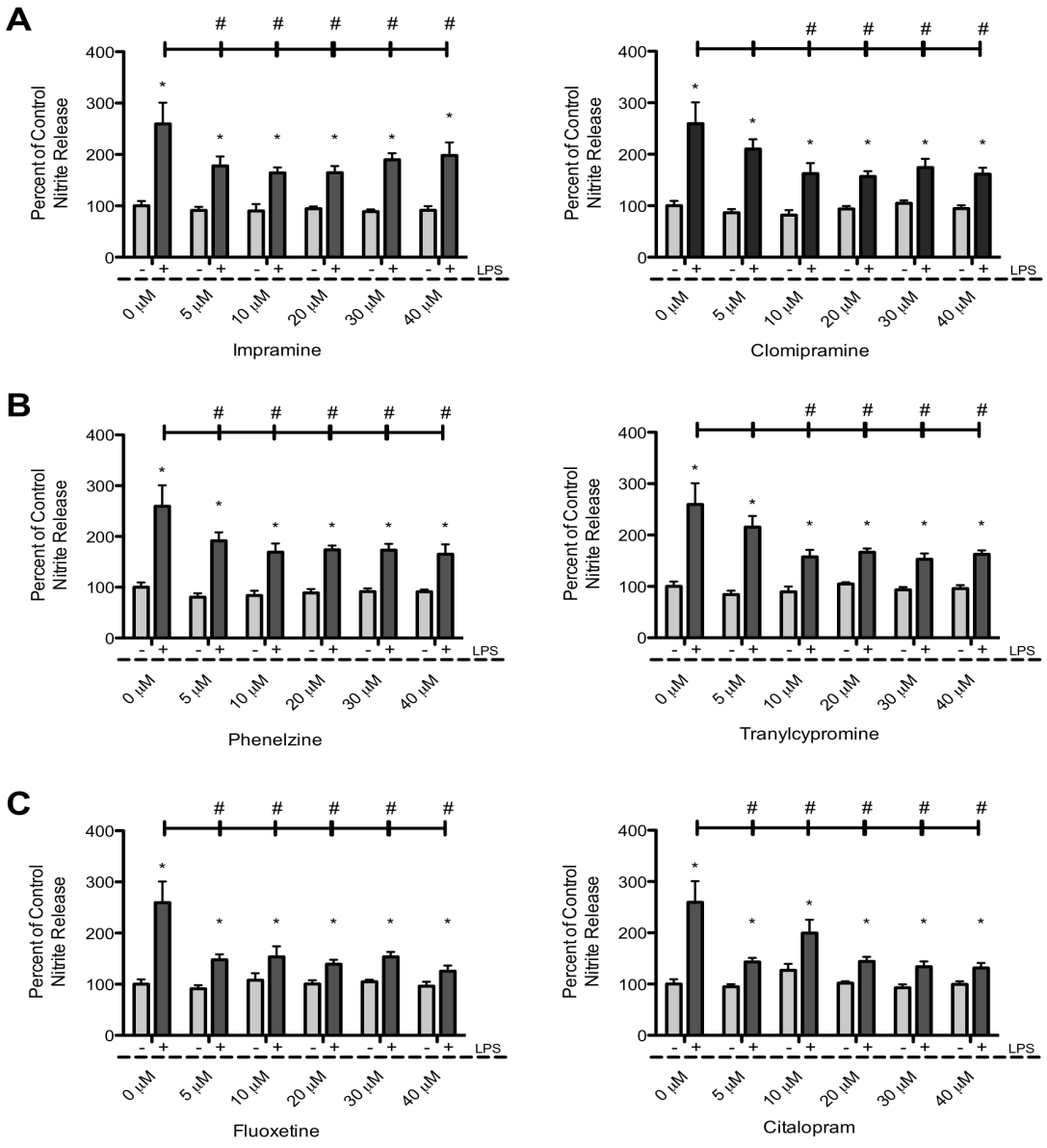


Figure 2-7 – NO release from resting and LPS-activated microglia treated with various doses of antidepressants following 24 hr treatment. **A.** All doses of the TCAs (imipramine and clomipramine), excluding 5 μM clomipramine, attenuated NO release from activated microglia. **B.** All doses of the MAOIs (phenelzine and tranylcypromine), excluding 5 μM tranylcypromine, attenuated NO release from activated microglia. **C**. All doses of the SSRIs (fluoxetine and citalopram) attenuated NO release from activated microglia. Antidepressants did not have an effect on NO release from resting microglia. * < 0.05 (compared within the same dose), #<0.05 (compared to LPS-treated microglia). LPS - 1 μg/ml.

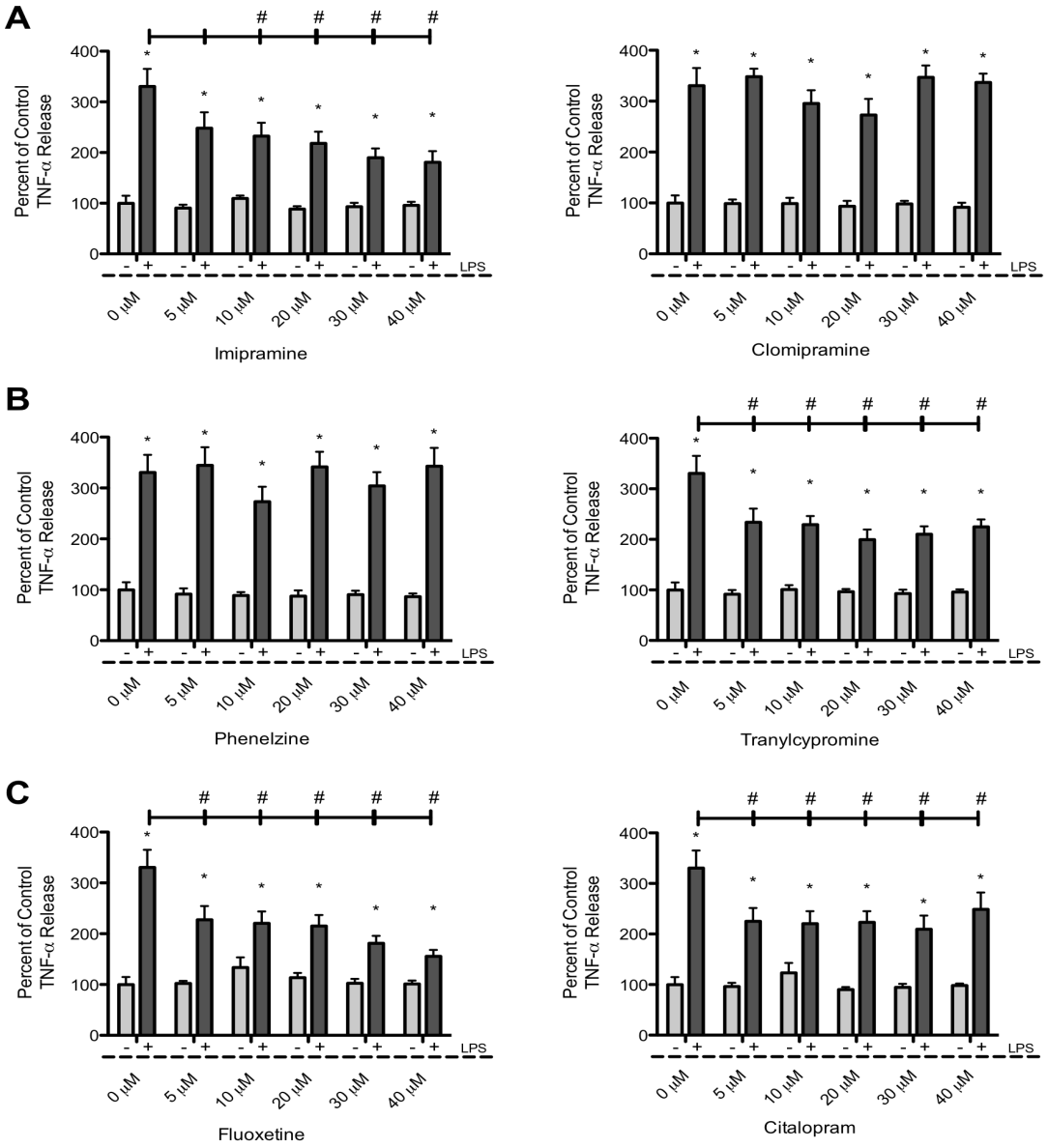


Figure 2-8 – TNF-α release from resting and LPS-activated microglia treated with various doses of antidepressants following 24 hr treatment. **A.** All doses of the TCA imipramine attenuated TNF-α release from activated microglia, but clomipramine had no effect. **B.** All doses of the MAOI tranylcypromine attenuated TNF-α release from activated microglia, but phenelzine had no effect. **C**. All doses of the SSRIs (fluoxetine and citalopram) attenuated TNF-α release from activated microglia. Antidepressants did not have an effect on TNF-α release from resting microglia. * < 0.05 (compared within the same dose), #<0.05 (compared to LPS-treated microglia). LPS - 1 μg/ml.

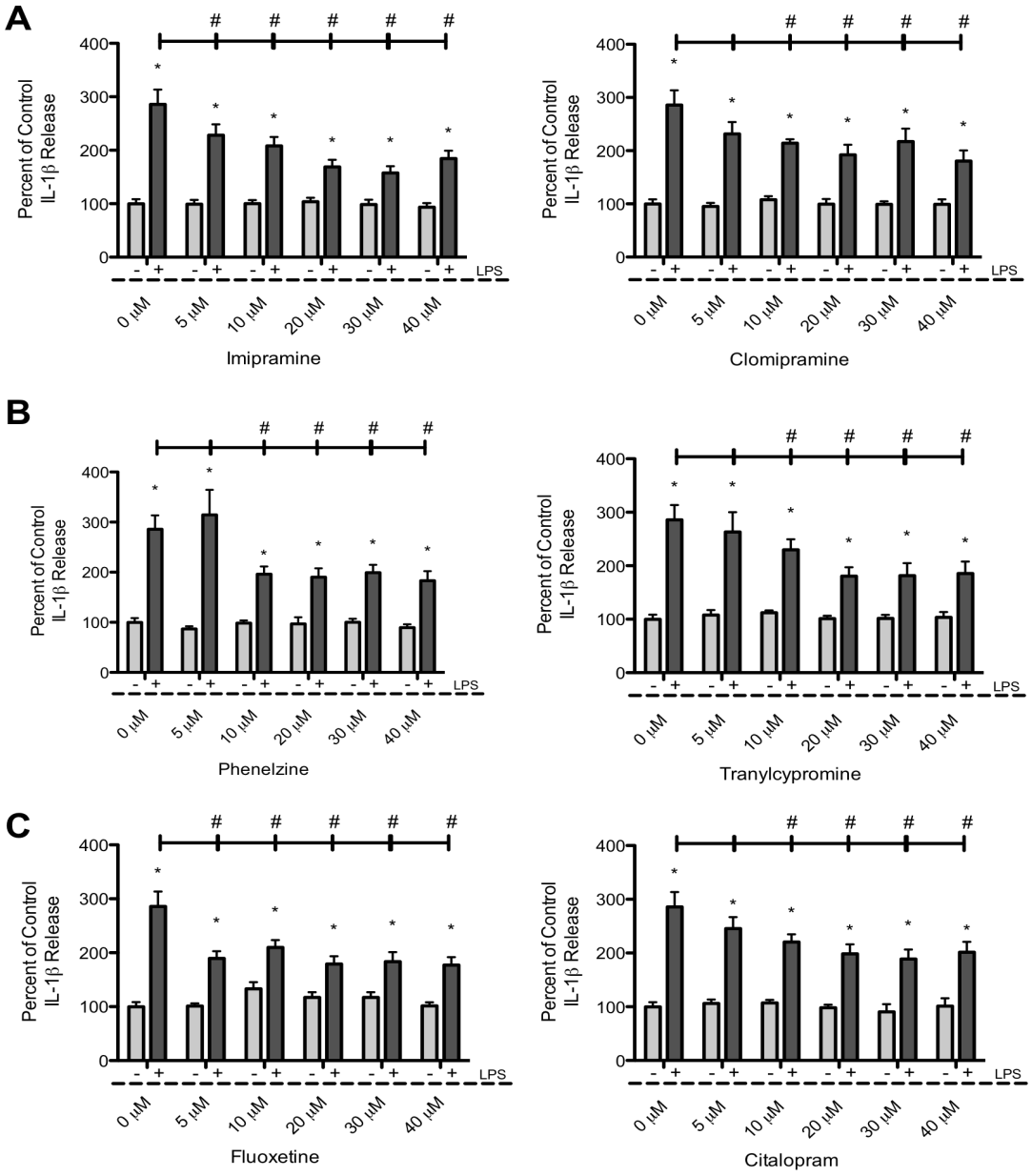


Figure 2-9 – IL-1β release from resting and LPS-activated microglia treated with various doses of antidepressants following 24 hr treatment. **A.** All doses of the TCAs (imipramine and clomipramine), excluding 5 μM clomipramine attenuated IL-1β release from activated microglia. **B.** All doses of the MAOIs (phenelzine and tranylcypromine), excluding 5 μM phenelzine and tranylcypromine attenuated IL-1β release from activated microglia. **C**. All doses of the SSRIs (fluoxetine and citalopram) excluding 5 μM citalopram attenuated IL-1β release from activated microglia. Antidepressants did not have an effect on IL-1β release from resting microglia. * < 0.05 (compared within the same dose), #<0.05 (compared to LPS treated microglia). LPS - 1 μg/ml.

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CHAPTER 3

Antidepressant effects on the viability of ischemic-injured neurons through the attenuation of microglial activation

3.1. Introduction

3.1.1. Ischemic stroke

Ischemia is the major type of stroke that occurs in the human population (Donnan et al. 2008). Ischemia is characterized by the restriction of blood flow to a region of the brain, resulting in a decrease in oxygen and glucose transport to brain tissue. In adults, ischemia can be the result of several conditions including thrombosis, embolism, or vasoconstriction. Thrombosis is the blockage of a blood vessel due to the formation of a clot (Ahmed et al. 2012). A blood clot that breaks free from its origin and circulates in the bloodstream to cause blockages in distal regions in blood vessels is referred to as an embolism (Fabijanic et al. 2012). Vasoconstriction is the narrowing in the diameter of a blood vessel, limiting the flow of blood to a region of the brain. No matter the cause in ischemia, the resulting lack of oxygen and glucose can lead to deficiencies in cellular metabolism and ultimately cellular death.

The middle cerebral artery (MCA) is the most common site of blood restriction causing ischemic stroke (Vakili et al. 2012). The MCA receives blood from the internal carotid artery and transports it to a number of regions in the brain including the basal ganglia, the insula, and large portions of the cortex; therefore, blockage of the MCA can lead to neuronal death in these regions (Coyle 1982). We chose to study specifically cortical neurons since these cells are highly susceptible to degeneration after a MCA occlusion (Hossmann 1988), and to our knowledge, no study has investigated cortical neuronal viability after an ischemic insult when co-cultured with microglia.

3.1.2. Correlation between depression and stroke

Depression and stroke are highly correlated to one another. A history of depressive episodes increases the risk of stroke in later life and the incidence of stroke increases the risk of future depressive episodes in patients (Esparrago Llorca et al. 2012). The most commonly prescribed treatment for pre- or post-stroke depression is antidepressants (Sunami et al. 2012); therefore, we were interested in determining if antidepressants affect the viability of neurons after an ischemic insult.

3.1.3. Antidepressant effects on the viability of injured neurons

Antidepressants have been shown to increase neuronal viability in various regions of the brain including the substantia nigra and the hippocampus (Jin et al. 2009; Kronenberg et al. 2012; Zhang et al. 2012); however, to my knowledge, no study has investigated the effects of antidepressants on cortical neurons in a model of ischemic stroke. To mimic ischemic conditions *in vitro*, cortical neurons can be deprived of oxygen by placing cell cultures in a hypoxic chamber within an incubator. Oxygen is displaced from the chamber by the infusion of nitrogen gas. In addition, cells are incubated in medium devoid of glucose. The lack of oxygen and glucose *in vitro* is collectively termed oxygen-glucose deprivation (OGD). After OGD insult to cortical neurons and their exposure to activated microglia, antidepressants may mediate the viability of injured neurons directly or indirectly. The term direct implies that the action of antidepressants is on the injured neurons, whereas the term indirect implies that antidepressants influence another cell type (e.g. microglia), which in turn mediates the viability of injured neurons. As discussed in chapter 2, several antidepressants attenuate the release of pro-

inflammatory factors from microglia and have been shown to have indirect effects on neuronal viability following injuries in the hippocampus and substanta nigra mediated through microglial release factors. Therefore, we investigated if antidepressants could affect cortical neuronal viability after neurons were given an ischemic insult and exposed to activated microglia; in addition, we investigated if this effect was mediated through microglial release factors.

To investigate our question, we required a special co-culture setup that incorporated the presence of microglia and cortical neurons in the absence of cell-to-cell contact between the distinct cells. In this setup, we could determine if the factors released from activated microglia cause further damage to injured cortical neurons and whether activated microglia pre-treated with antidepressants attenuate this damage. For a schematic of the co-culture refer to **Figure 1A**.

3.1.4. Measuring cell viability

Cellular viability in culture can be determined by measuring various cellular properties including cell membrane leakage and mitochondrial activity. Various dyes such as propidium iodide and tryphan blue can be utilized to determine membrane leakage (Malagolini et al. 2013; Motawi et al. 2013). In addition, lactate dehydrogenase, a stable enzyme within healthy cells, is released into the media after a cell membrane is compromised (Karahashi et al. 2009). To measure mitochondrial activity, the most common test is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Nicholas et al. 2001; Capela et al. 2013). In active cells, MTT is reduced to purple

formazan via NADPH-dependent cellular enzymes, whereas compromised cells have a decrease in this reduction of MTT. By dissolving the end product with a strong detergent, the absorbance values can be quantified to determine overall activity in a population of cells.

3.1.5. Chapter objectives

The first step in this study was to select a suitable time of OGD for cortical neurons so that approximately a 40-50% loss in cell viability occurred prior to placing these neurons in co-cultures with microglia. Once in co-culture, we can determine if antidepressant-treated activated microglia can increase or decrease neuronal viability compared to the initial 40-50% decline in viability. We hypothesized that activated microglia would further decrease the viability of OGD-injured neurons through the release of pro-inflammatory factors and that the pre-treatment of microglia with antidepressants would attenuate this decrease.

3.2. Materials and methods

3.2.1. Primary mixed glial cultures

Mixed glia were prepared from whole brains of post natal day 1 Sprague-Dawley rats. For detailed descriptions of the dissociation methods, refer to **Section 2.2.1.**

3.2.2. Primary microglial culture

After 21 DIV, microglia were isolated from mixed glial cultures via trypsinization (Saura et al. 2003). For a detailed description of the trypsinization method, refer to **Section 2.2.2.**

3.2.3. Primary cortical neurons

Cortical neurons were cultured from embryonic Sprague-Dawley day 18 rats based on previous methods (Brewer et al. 1993; Swayze et al. 2004; Lai and Todd 2006). Cortices were dissociated in 0.25% trypsin-EDTA for 25 min, followed by mechanical triturating in DMEM/F-12 with 10% FBS and 2% P/S; FBS was used to saturate the enzymatic activity of trypsin with excess protein. The cells were re-suspended in Neurobasal medium (Gibco) supplemented with 0.5mM L-glutamine, 2% penicillin-streptomycin and 2% B-27 (all from Gibco) and plated on poly-L-lysine-coated 24-well plates or inserts (6.5mm diameter, 0.4μM, Costar). Cell density was plated at 1.2 X 10^5 cells per insert or 2 X 10^5 cells per well in a 24 well plate for immunofluorescence. Contaminating glial cells were eliminated by treatment with 1 μM cytosine arabinoside (Sigma), an agent that kills proliferative cells, from 3 DIV to 6 DIV. Neurobasal medium was replaced with fresh medium every 3-4 DIV. Cortical cultures were used for experiments at 12 DIV.

3.2.4. Antidepressants/chemicals

Three classes of antidepressants were used to pre-treat non-activated and LPS-activated microglia prior to co-culture, namely TCAs (imipramine and clomipramine), MAOIs (phenelzine and tranylcypromine [TCP]) and SSRIs (fluoxetine and citalopram). LPS was chosen as a microglial activator.

3.2.5. Neuronal OGD insult

Medium was removed from neuronal 12-14 DIV cultured inserts and replaced either with fresh Neurobasal medium or glucose-free medium (Gibco). The neuronal inserts were then subjected to one of two conditions for various time points (0 min, 20 min, 30 min, 40 min, 50 min, and 60 min) at 37°C: normoxic (normal oxygen with Neurobasal media) or oxygen-glucose deprived (low oxygen [<1%] without glucose; OGD). After insult, the medium was removed and fresh Neurobasal medium was added and neurons were left to incubate for 24 hrs followed by the assessment of neuronal viability (**Section 3.2.6**).

3.2.6. Assessment of cortical neuron viability

Neuronal activity was assessed using the MTT colorimetric assay and was used as an indirect measure of cell viability. Briefly, 100µl of MTT (final concentration of 0.5 µg/ml) were added to the neuronal wells or inserts and left to incubate for 40 min at 37°C. Medium was aspirated and replaced with 210µl of dimethyl sulfoxide (DMSO) for cell lysis. Absorbance was read at 570nm on a plate reader (Powerwave X, Bio-Tek).

3.2.7. Immunocytochemistry

Primary cortical neurons were cultured on sterile coverslips (12 mm in diameter, Fisher) in 24 well plates. Cells were fixed with 10% formalin for 10 min. After 3 washes with PBS, the cells were incubated with a blocking solution [10% normal horse serum (Gibco) in PBS with 0.1% Triton X-100] for 1hr. After washing, cells were incubated with MAP-2 primary antibodies (diluted in PBS with 1% normal horse serum) overnight at 4°C. After 3 additional washes, wells were incubated with the relevant fluorescent-conjugated

secondary antibodies (Invitrogen) diluted in PBS for 1hr. After a final wash, wells were cover-slipped using aqueous mounting medium. Fluorescence-conjugated secondary antibodies (1:2000) were purchased from Jackson ImmunoResearch. Axiovision imaging software coupled to an Olympus fluorescent microscope was used to capture these images. Nine images were captured per well for each filter. Merged images were created by Image-J software.

3.2.8. Co-culture

Non-activated and LPS-activated microglia pre-treated with antidepressants were washed with Neurobasal medium four times to clear residual LPS or antidepressants and fresh Neurobasal medium was added to microglia. Antidepressants (10 μM) were added to microglia 30 min before LPS activation (1μg/ml) and the cultures were left to incubate for 24 hrs. Microglial cultures were randomly assigned to one of the following four conditions: control (PBS only), LPS-treated, antidepressant-treated, and LPS + antidepressant-treated. To demonstrate the washing away of residual LPS from microglial cultures, we transferred washing medium following each wash (4 washes) to a new set of microglial cultures for 24 hrs and then measured NO release in the medium.

Inserts with injured or healthy neurons were placed into microglial wells so that they were incubating in the medium but not in direct contact with microglia. As a control, inserts were placed into wells that contained no microglia. The co-cultures were left to incubate for 24 hrs at 37°C with 5% CO_2. The following day, neuronal viability was quantified with the MTT assay. For a detailed schematic of the experimental protocol,

refer to **Figure 1 B**. To assess any direct effects of antidepressants on neuronal viability after an injury, cortical neurons cultured on inserts were placed under OGD or normoxic conditions for 40 min. After 40 min incubation, medium was replaced with fresh Neurobasal medium and antidepressants (10μM) were added to the neurons, which were incubated for 24 hrs and neuronal viability was determined with the MTT assay.

3.2.9. Statistical Analysis

Significance levels for data sets were calculated using a two-tailed t-test (designated with *) or a two way ANOVA followed by Bonferroni *post hoc* test (designated with #). t-Tests were used to compare two data sets involving the manipulation of only one variable. Two way ANOVAs were used to compare two data sets involving manipulations of two or more variables. Both designations * and # denote $p < 0.05$. Refer to figure legends for total 'n' values for specific experiments. For all MTT absorbance values, each 'n' represents one insert of a neuronal culture and inserts were taken from a minimum of three separate culture preparations for each study.

3.3. Results

3.3.1. Primary cortical neurons were not contaminated with astrocytes or microglia

Before utilizing the cultured primary cortical neurons in the co-culture setup, we investigated if the cultures contained purely neurons. We immuno-labeled with the neuronal marker MAP-2, and, as expected, found that there were neurons in the culture, whereas labeling of the cells with the astrocyte and microglia markers, GFAP and Iba1 respectively, yielded no expression (**Figure 3-2**). These results demonstrated that the

cultured primary cortical neurons are pure and are not contaminated with astrocytes and microglia, two major types of mitotic glial cells.

3.3.2. OGD injury for 40 min reduced neuronal viability by approximately 40%

Our next goal was to investigate the time of OGD injury required to reduce primary cortical neuronal viability to approximately 40-50 % compared to healthy neurons by measuring MTT absorbance, a measure of cell activity. We found that OGD injury times of 20 and 30 min decreased MTT absorbance values by 15% and 23%, respectively, but these decreases were not significant (**Figure 3-3 A**). OGD injury for 40, 50, and 60 min yielded significant decreases in MTT absorbance by 40%, 65%, and 80 % respectively (**Figure 3-3 A**). Immuno-labeling of cortical neurons with MAP-2 revealed losses in neuronal branches after 40, 50, and 60 min of OGD injury compared to 0, 20, and 30 min of injury (**Figure 3-3 B**). Observations comparing 0, 20, and 30 min showed little to no changes in neuronal branches (**Figure 3-3 B**). Observations following immuno-labeling with MAP-2 showed that cortical neurons were likely undergoing cellular death following OGD-insults at time points 40, 50, and 60 min and that these decreases in MTT absorbance were not a result of a decrease in mitochondrial activity in healthy cells. We demonstrated this by counting the number of branches per neuronal cell in the photomicrogaphs and found that neurons that had undergone OGD for 40, 50, and 60 min had significantly lower numbers of branches per cell compared to 0, 20, and 30 min of injury (**Figure 3-3 C**). Since decreases in MTT absorbance values strongly correlated with a lower number of neuronal branching per cell following OGD injury, we were confident in using MTT absorbance as the measure of neuronal viability after co-culture.

3.3.3. LPS-activated microglia continue to release NO and TNF-α for 24hrs following LPS removal

Prior to the co-culture of cortical neurons with microglia, non-activated and LPS-activated microglia are washed to remove LPS and then these microglia are placed into a co-culture. LPS is removed to rule out its direct effects on neuronal viability. In the absence of LPS, we needed to demonstrate that microglia remained activated for 24 hrs following its removal, since during this time interval these microglia are in co-culture with cortical neurons. As a measure of activation, NO and TNF-α release from microglia was quantified 24 hrs following LPS removal. We found that following LPS removal release of both NO and TNF-α was significantly increased compared to non-stimulated controls at both 24 hrs and 48 hrs (**Figure 3-4 A, Figure 3-4 B**). There were no differences in the release of NO or TNF-α from microglia 24 hrs following LPS removal compared to LPS-activated microglia at 24 hrs (**Figure 3-4 A, Figure 3-4 B**). Based on these results, microglia remain activated 24 hrs post LPS removal as these cells continue to release pro-inflammatory factors such as NO and TNF-α. We also demonstrated the removal of LPS from microglial cultures after washing. LPS-activated and non-activated microglia were washed 4 times with medium to remove LPS. After each wash, these media were transferred to a new set of microglial culture for 24hrs. After the 24 hrs, we measured the amount of NO released from the new set of cultures. If residual LPS remained after the 4^{th} wash, then this washing medium would contain LPS, and activate a new set of cultures. We found that the transfer of medium from the 1^{st} wash but not the 2^{nd}, 3^{rd}, or 4^{th} washes to a new set of microglial culture increased the release of NO

compared to controls (**Figure 3-4 C**). This result demonstrates that after the 4th washing of microglial cultures there is no residual LPS left.

3.3.4. Antidepressants have no direct effect on the neuronal viability of OGD injured or healthy cortical neurons

The next goal was to investigate if antidepressants could affect the viability of healthy or OGD-injured cortical neurons. We cultured cortical neurons on inserts and found that when cortical neurons were pre-treated with any of the antidepressants tested, there was no effect on neuronal viability following OGD injury compared to conditions with no drug added; in addition, none of the antidepressants tested had a direct effect on the viability of healthy neurons (neurons not exposed to OGD) (**Figure 3-5 A**). In summary, the antidepressants tested do not affect the viability of healthy or OGD-injured neurons in culture.

3.3.5. Activated microglia in co-cultures with OGD-injured cortical neurons further decrease neuronal viability

Next, we investigated if the presence of non-activated or LPS-activated microglia in co-culture had an effect on the viability of healthy or OGD-injured neurons. We found that there was no difference in the viability of healthy or OGD-injured neurons when placed in co-culture with or without the presence of non-activated microglia (**Figure 3-5 B**). Therefore, the presence of non-activated microglia does not affect the viability of healthy or OGD-injured neurons in co-culture. When OGD-injured neurons were placed in a co-culture with LPS pre-activated microglia, we found that the viability of neurons

significantly decreased compared to that of injured neurons in co-cultures with non-activated microglia (**Figure 3-5 B**). When healthy neurons (no OGD injury) were placed in co-cultures with LPS pre-activated microglia, there was a small decrease in the viability of neurons compared to the viability of healthy neurons not placed in co-cultures; however, this decrease was not significant (**Figure 3-6**). Therefore, OGD-injured neurons are vulnerable to a further decrease in viability when placed in a co-culture with microglia pre-activated with LPS; however, healthy neurons do not show this vulnerability to pre-activated microglia.

3.3.6. Pre-treatment of activated microglia with fluoxetine and citalopram attenuates the further decrease in viability of OGD-injured neurons in co-culture

The final study in this chapter was to investigate if antidepressants could affect the viability of OGD-injured neurons in co-cultures by attenuating the profile of LPS-activated microglia. We found that when microglia were pre-treated with imipramine, phenelzine, or TCP and LPS for 24 hrs and then added to co-cultures for an additional 24 hrs with OGD-injured neurons, there were slight increases in the viability of neurons compared to those co-cultures with non-treated microglia; however, these increases were not significant (**Figure 3-6**). When microglia were pre-treated with fluoxetine or citalopram and LPS for 24 hrs and added to co-cultures for an additional 24 hrs with OGD-injured neurons, there was a significant increase in the viability of neurons compared to those co-cultures with non-treated microglia (**Figure 3-6**). All conditions involving pre-treated microglia with antidepressants and LPS for 24 hrs failed to increase the viability of OGD-injured neurons to the levels of healthy neurons (not exposed to

OGD) after an additional 24 hrs in co-culture. Therefore, activated microglia pre-treated with fluoxetine or citalopram attenuate the further decrease in the viability of OGD-injured neurons in co-culture but this increase does not reach the viability levels of non-injured healthy neurons.

3.4. Summary

In this chapter, we accomplished a number of goals that lead to further questions and experiments in this thesis. First, we needed to determine a length of time of OGD injury to which to expose cortical neurons. We required a time that caused approximately a loss in 50% viability compared to healthy controls. We found that cortical neurons exposed to OGD for 40 min caused a 40% loss in viability compared to healthy controls, and thus used this time of OGD as the injury model of cortical neurons in this thesis. MTT absorbance values were corroborated immunofluorescent images showing losses in neuronal projections at 40, 50, and 60 min after OGD exposure.

The next goal was to see if antidepressants could attenuate the loss in neuronal viability following OGD injury. We found that pre-treating OGD-injured neurons with these antidepressants had no effect on the viability of neurons; thus there was no direct effect of antidepressants on neurons. In control conditions, the presence of non-activated microglia in co-culture had no effect on the viability of OGD-injured neurons and pre-activated microglia had no significant effect on the viability of healthy neurons (no OGD insult). When OGD-injured neurons were in the presence of pre-activated microglia, there was a further reduction in viability and this reduction was attenuated when these

pre-activated microglia were also pre-treated with the SSRIs (fluoxetine and citalopram). The other antidepressants investigated in this thesis did not have this attenuating effect.

The results in this chapter lead to further questions that we attempt to answer in chapter 4. We found the majority of antidepressants in this thesis attenuate the release of the pro-inflammatory factors (NO and TNF-α) from activated microglia (**Chapter 1**), but we found with activated microglia that pre-treatment with only the SSRIs (fluoxetine and citalopram) attenuated the further decrease in viability of injured neurons. If the release of NO and TNF-α from activated microglia was the key determinant in reducing the viability of injured neurons, then we would expect the other antidepressants to also attenuate this loss in viability; however, this is not what we observed. Therefore, we hypothesized that LPS-activated microglia release factors other than the ones already discussed, and that are key players in reducing the viability of injured neurons. In addition, we hypothesized that fluoxetine and citalopram will be the only antidepressants of the ones tested to attenuate the release of these key players from microglia. The next chapter investigates these hypotheses.

3.5. Figures

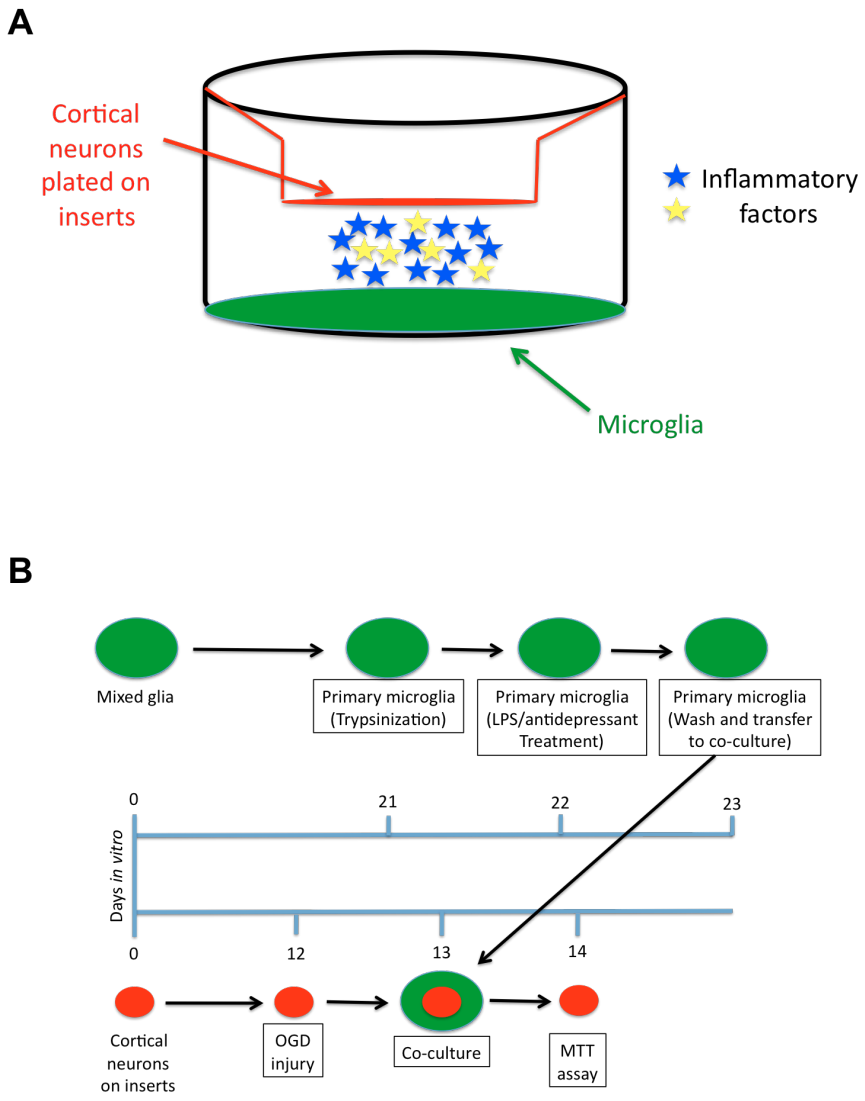

Figure 3-1 - Schematics of the co-culture and experimental setups. A. Co-culture setup consisting of primary microglial cells (green) plated in 24-well plates and primary cortical neurons (red) plated on inserts. Microglia and neurons are not in contact with one another but factors released from cells can contact both cell types. **B.** Experimental setup displaying the co-culture timeline.

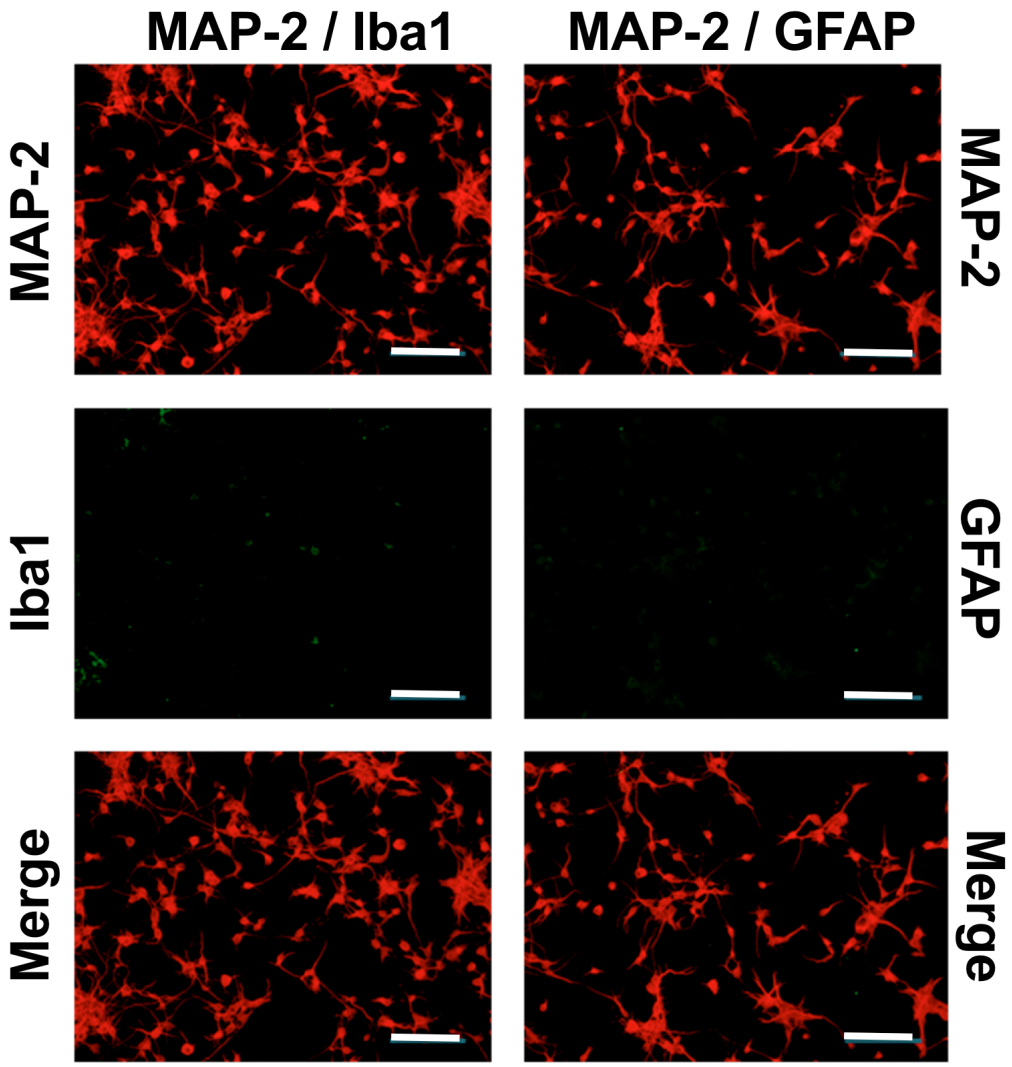

Figure 3-2 - Immunostaining for MAP-2 (neurons, red), Iba1 (microglia, green), and GFAP (astrocytes, green) to verify purity of primary cortical neuronal cultures. Top row: Cultures with MAP-2-expressing neurons (red); Middle row, Iba1GFAP staining for astrocytes (green); Right, merged image. (D) Left, MAP-2 staining for neurons (red); Middle, Iba-1 staining for microglia (green); Right, merged image. All images X 200 magnification. Scale bars = 80 μm.

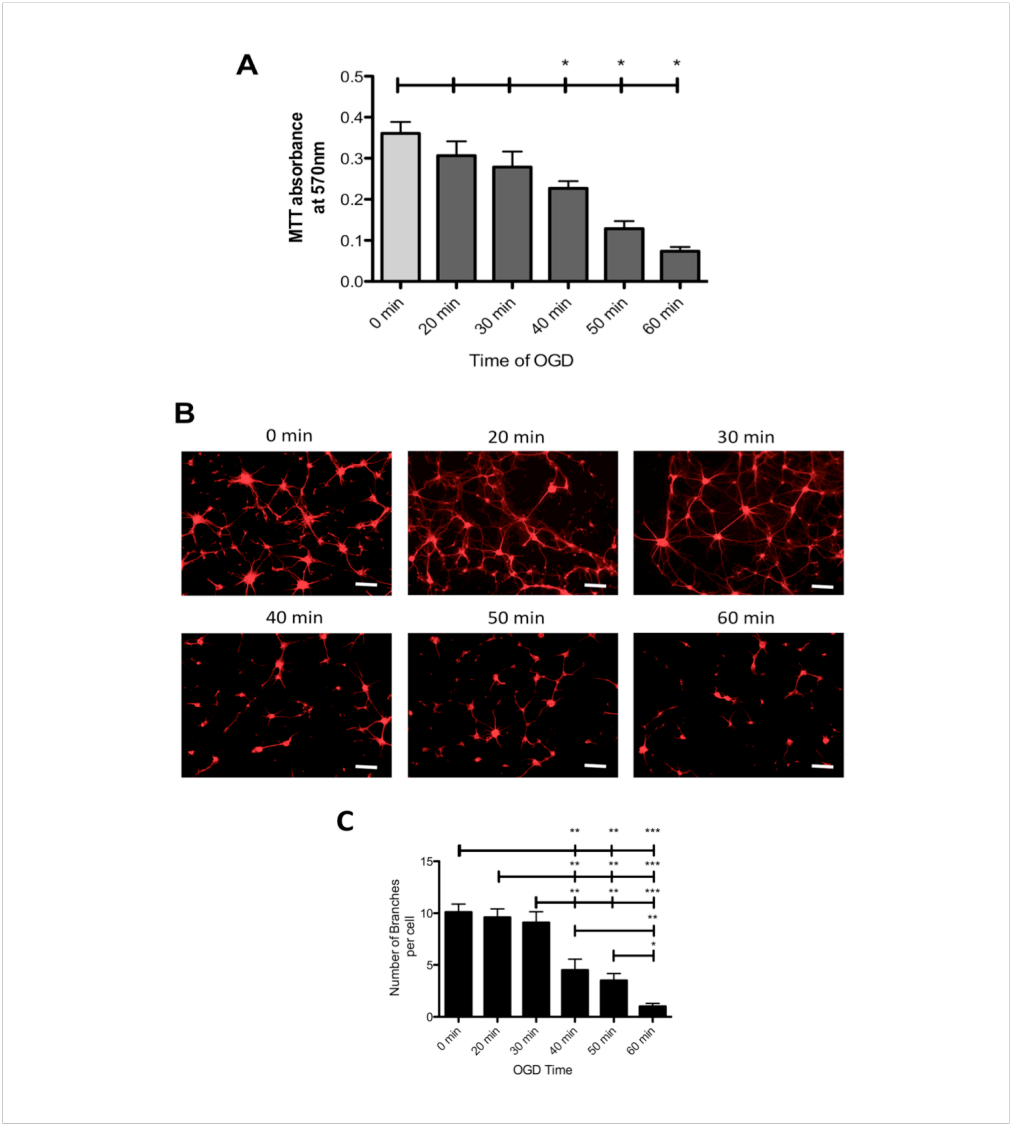

Figure 3-3 – MTT quantification and immuno-fluorescent photomicrographs of cortical neurons after different times of OGD injury. A. OGD at time points of 40, 50, and 60 min significantly decreased MTT absorbance values compared to 0 min (control); OGD time points at 20 and 30 min had no significant effect; n = 10. **B.** Photomicrographs showing MAP-2 immuno-fluorescence of cortical neurons after various time points of OGD injury. **C.** Number of branches per neuronal cell decreases at the OGD time points 40, 50, and 60 min. Photomicrographs and the number of branches per cell correlate to MTT absorbance values at all OGD time points. Scale bar = 150 μM, 20X objective.

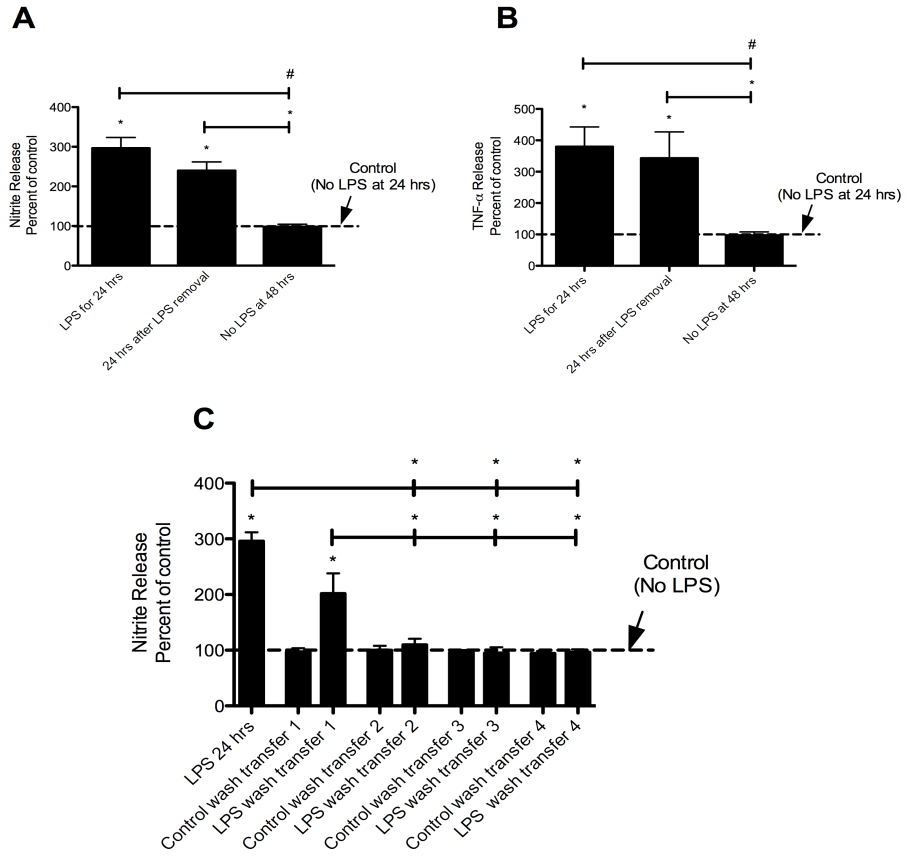

Figure 3-4 – The release of NO and TNF-α 24 hrs following LPS removal from microglial cultures. **A**. The effect of LPS removal on NO release from activated microglia. LPS activation for 24 hrs significantly increases NO release from microglia compared to non-activated controls. After washing out the cultures to remove LPS, the microglia continued to release NO for an additional 24 hrs. Non-activated controls at 48 hrs showed no differences in NO release compared to non-activated controls at 24 hrs, n = 8. **B.** The effect of LPS removal on TNF-α release from activated microglia. LPS activation for 24 hrs significantly increases TNF-α release from microglia compared to non-activated controls. After washing out the cultures to remove LPS, the microglia continued to release TNF-α for an additional 24 hrs. Non-activated controls at 48 hrs showed no differences in NO release compared to non-activated controls at 24 hrs, n = 8. **C.** Demonstrating the removal of LPS from microglial cultures by washing. LPS-activated microglia were washed 4 times with DMEM to remove LPS and these wash media were added to a new microglial culture for 24hrs. Following the addition of medium from the 1st wash but not the 2nd, 3rd, or 4th washes to new microglial cultures increased the release of NO compared to controls. Statistical significance, * < 0.05 (compared to control at 24 hrs), #<0.05 (comparing the LPS 24 hr group to the no LPS 48 hr group).

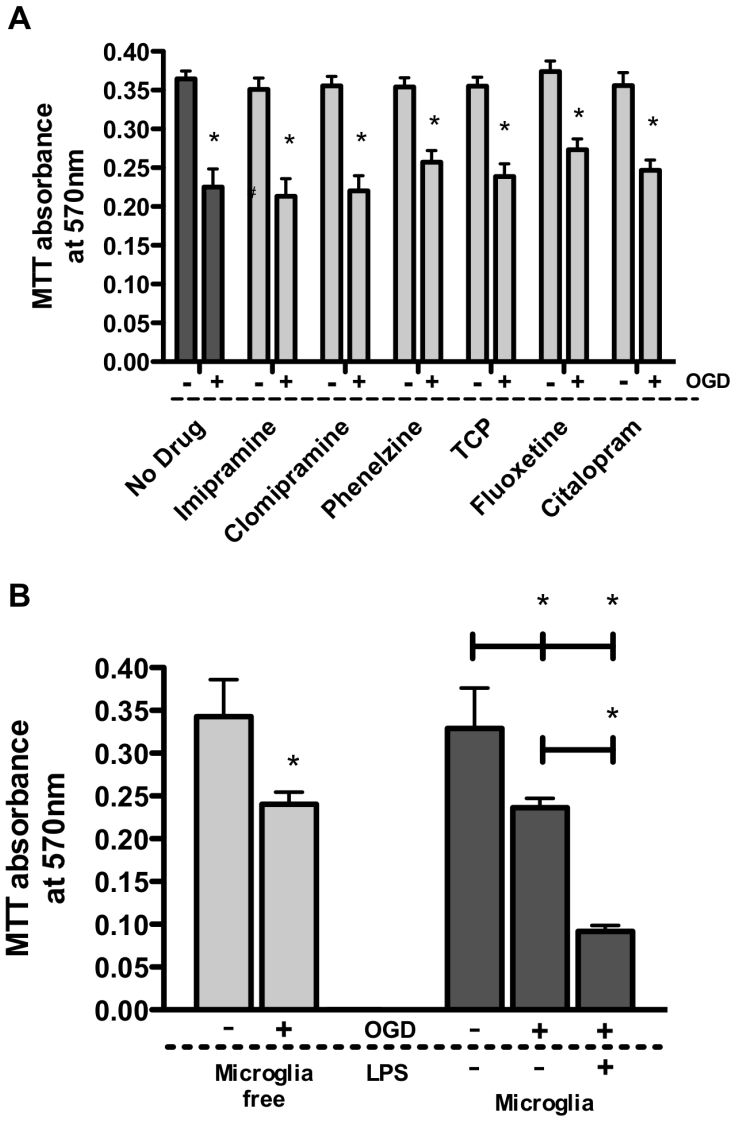

Figure 3-5 – Control studies measuring cortical neuronal viability by MTT absorbance. **A**. The effect of antidepressants on the viability of cortical neurons before and after OGD injury. Antidepressants had no effect on cortical neuronal viability when treated on normoxic or OGD neurons for 24 hrs, n = 10. **B.** The effect of non-activated and LPS-activated microglia on cortical neurons viability after OGD injury. MTT absorbance was measured 24 hrs after co-culture. The presence or absence of microglia had no effect on OGD-injured or normoxic cortical viability after 24 hrs of co-culture. Pre-treatment of microglia with LPS prior to 24 hrs of co-culture significantly reduced cortical neuronal viability after OGD injury, n = 10. Significance of $p < 0.05$ is represented with *, within a group.

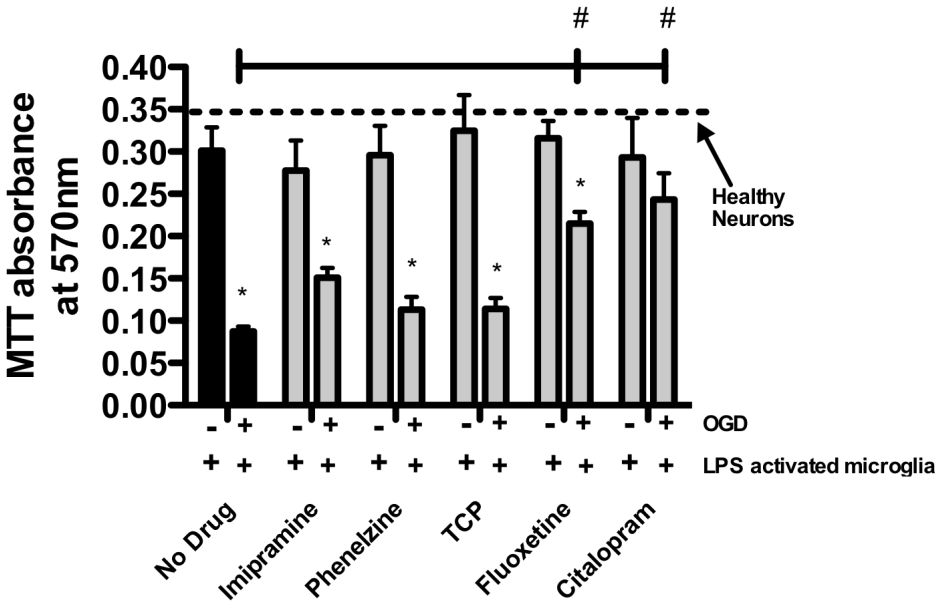

Figure 3-6 – The effect of microglia pre-treated with antidepressants on cortical neuronal viability after OGD injury. Healthy and OGD-injured cortical neurons were placed in co-cultures with antidepressant-treated LPS-activated microglia for 24 hrs. The pre-treatment of LPS-stimulated microglia with fluoxetine and citalopram significantly increased the viability of injured neurons compared to the no drug group, whereas imipramine, phenelzine, and TCP had no effect, n = 10. LPS-activated microglia slightly decreased viability of healthy neurons (no OGD injury, dashed line), but the change was not significant. Comparisons were made with t-test or two-way ANOVA followed by Bonferroni post-test where appropriate. *P < 0.05 (comparisons within a drug group), #P < 0.05 (comparisons across drug groups).

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CHAPTER 4

Determining the main factors derived from activated microglia involved in mediating the viability of ischemia-injured neurons

4.1. Introduction

4.1.1. Glutamate

Chapter 3 showed that the pre-treatment of activated microglia with fluoxetine and citalopram attenuated the further decrease in the viability of OGD-injured cortical neurons in co-culture. However, it is unclear as to which mediators released from activated microglia are the key players in decreasing the viability of these neurons. Pro-inflammatory factors such as NO and TNF-α do not seem to be these key players since the majority of antidepressants we tested attenuated their release from activated microglia, but failed to significantly increase the viability of injured neurons in co-culture. Therefore, there must be other relevant factors released from activated microglia.

In addition to inflammatory mediators, activated microglia can release amino acids and trophic factors (Patrizio and Levi 1994; Miwa et al. 1997; Wu et al. 2004; Chen et al. 2012; McMullan et al. 2012). For instance, the amino acid glutamate, the most abundant excitatory neurotransmitter in the CNS (Meldrum 2000), is released from microglia following activation with LPS (Casamenti et al. 1999; McMullan et al. 2012). Glutamate is released from pre-synaptic neurons and stimulates AMPA and NMDA receptors on post-synaptic neurons, allowing an influx of sodium and calcium into the post-synaptic cell to depolarize the cell and help propagate a chemically induced signal in the form of an action potential down the axon. Glutamate is essential for the normal functioning of the CNS, but the accumulation of glutamate can have detrimental effects on neurons through excitotoxicity (Campos et al. 2012; Lee et al. 2012) resulting from the over-

stimulation of NMDA receptors, leading to the influx of excess calcium into neurons. This internal rise in calcium concentrations causes mitochondrial damage and the induction of pro-apoptotic transcription factors, eventually leading to cell death (Cheng et al. 2012; Zadori et al. 2012).

4.1.2. The NMDA receptor

NMDA glutamate receptors are complexes made up of four subunits derived from three related families: NR1, NR2, and NR3 subunits (Mori and Mishina 1995). A single NMDA receptor most often contains two NR1 subunits and two NR2 subunits (Laube et al. 1998). The NR1 subunit contains the glycine or D-serine binding site (Hirai et al. 1996), whereas the NR2 subunit contains the glutamate binding site (Laube et al. 1998). A glycine or D-serine responsive excitatory receptor that does not require glutamate is formed when the NR3 subunit complexes with the NR1 subunits (Chatterton et al. 2002). There are several binding sites on the NMDA receptor for other modulators, including the drugs phencyclidine, MK-801, and memantine, all of which are NMDA channel blockers (Anis et al. 1983; Gill et al. 1987). Zinc binding to the NMDA receptor causes a voltage-dependent reduction in NMDA receptor responses in NR1/NR2 complexes and a reduction in channel opening probability (Chen et al. 1997). Polyamine binding to the NMDA receptor increase the affinity of glycine binding to the receptor and potentiates responses of the NMDA receptor even after glycine saturation (Sacaan and Johnson 1989). The first full antagonist discovered for the glycine binding site was kynurenic acid (Kessler et al. 1989), and structural modification of kynurenic acid led to a series of additional potent antagonists including L-701,324 (L701) (Kulagowski et al. 1994).

4.1.3. Glycine and D-serine

Glycine and D-serine are other amino acids implicated in excitotoxicity. Glycine is an inhibitory neurotransmitter in the spinal cord and brainstem by allowing chloride to enter neurons through ionotropic receptors. In cortical regions of the brain, glycine is a co-agonist along with glutamate at NMDA receptors (Liu and Zhang 2000). Low glycine levels can indirectly activate glia to release D-serine (Miraucourt et al. 2011). D-Serine acts as a co-agonist on NMDA receptors and shows approximately a three-fold greater potency than glycine at the same site (Matsui et al. 1995; Wolosker 2007). In addition, microglia can release D-serine after amyloid β-peptide stimulation *in vitro* (Wu et al. 2004).

There is a paucity of drugs available that inhibit the glycine and D-serine co-agonist site on the NMDA receptor. One drug that has been extensively used for *in vivo* experiments is L-701, 324 (L701); however, to our knowledge, this drug has not been used for *in vitro* experiments (Murray et al. 2011; Wlaz and Poleszak 2011). Therefore, the beginning of this chapter will focus on the ability of L701 to inhibit the glycine and D-serine site on the NMDA receptor *in vitro*. After demonstrating that L701 has its inhibitory effect in culture, we can then utilize the drug in future experiments.

4.1.4. Gamma-aminobutyric acid (GABA)

GABA is another amino acid that has been shown to be released from activated microglia and astrocytes (Casamenti et al. 1999; Lee et al. 2011). The majority of research investigating the glial release of GABA focuses on the spinal cord rather than the brain.

A reduction in GABA levels within the spinal cord is one factor responsible for the emergence of neuropathic pain, leading to the decline in spinal dorsal horn inhibition and greater excitability of sensory neurons (Zeilhofer 2008). In addition, the MAOI phenelzine can increase GABA levels within the central nervous system of rodents with experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (Musgrave et al. 2011).

4.1.5. Taurine

Taurine is an essential amino acid for brain development in humans and rodents (Sturman et al. 1985). In NPC cultures from adult mice, taurine stimulates the proliferation and promotes the neurogenesis of these cells (Hernandez-Benitez et al. 2012). Taurine deficiency leads to a decrease in the development, mitosis, migration, and organization of cells in the brain of rodents (Sturman et al. 1985). The highest levels of taurine in the brain are found in the olfactory bulb, a region that constantly receives proliferating NPCs from the sub-ventricular zone, and taurine levels remain elevated in the olfactory bulb compared to other regions of the brain during brain maturation (Miranda-Contreras et al. 2000). It is unclear how taurine stimulates cell proliferation, but one idea is that taurine prevents imbalances in redox species within NPCs, making the environment within the cells favorable for proliferation (Sharma et al. 2008). In addition to promoting neurogenesis and proliferation of NPCs, taurine can protect neuronal cultures against hypoxic-induced death and has been found to have anti-inflammatory properties on microglia (Ward et al. 2011; Pan et al. 2012).

4.1.6. Glutamine

Glutamine is an important amino acid in excitotoxicity since its levels in the brain are linked to glutamate levels. Glial (i.e. astrocytes and microglia) take up excess glutamate in the extrasynaptic space and convert this into glutamine with the enzyme glutamine synthetase (Martinez-Hernandez et al. 1977). Glutamine is then released from these glia and taken up by neurons where it is converted back into glutamate by the mitochondrial enzyme glutaminase (Daikhin and Yudkoff 2000). In addition to neurons, activated macrophages can up-regulate their expression of glutaminase and increase the activity of glutamate synthesis (Tian et al. 2008; Erdmann et al. 2009). This excess glutamate can be released from activated macrophages and cause excitotoxic effects on neurons. When glutaminase activity is attenuated by an inhibitor (6-diazo-5-oxo-L-norleucine), glutamate production in activated macrophages is decreased, lending to reduced neurotoxic effects (Yawata et al. 2008).

4.1.7. The release of trophic and anti-inflammatory factors from activated microglia

Microglia can alter the release of trophic and anti-inflammatory factors such as BDNF, GDNF, and IL-4 after activation. Activated microglia increase the release of BDNF in the spinal cord and can lead to neuropathic pain, whereas increased release in the brain can lead to neurogenesis and cell survival (Coull et al. 2005; Beggs et al. 2012; Trang et al. 2012). GDNF is another trophic factor released from microglia, but unlike BDNF, the activation of microglia with LPS in culture suppresses the release of GDNF (Matsushita et al. 2008). Finally, IL-4 has been shown to have anti-inflammatory effects in the brain by reducing motoneuron degeneration induced by activated microglia (Zhao et al. 2006;

Cao et al. 2007). However, there is a lack of studies that have investigated the release of IL-4 from non-activated and activated microglia.

4.1.8. Chapter objectives

In this chapter, we attempted to determine the key players released from activated microglia that cause the further decrease in viability of OGD-injured cortical neurons in co-culture. The candidates include various amino acids (glutamate, D-serine, glycine, glutamine, and taurine), trophic factors (BDNF and GDNF), anti-inflammatory factors (IL-4), or pro-inflammatory factors (TNF-α and NO). Our first goal was to measure the release of the various amino acids, trophic factors, and IL-4 from non-activated and LPS-activated microglia following antidepressant treatments. Next, we used various antagonists against the factors released from activated microglia to determine if there are any differences in the viability of OGD-injured neurons in co-culture once a specific factor is neutralized. Prior to the antagonism studies, we performed various control experiments to demonstrate that the antagonists execute their inhibitory or scavenging functions *in vitro*. We hypothesized that the key players released from microglia in decreasing the viability of OGD-injured neurons in co-culture are likely glutamate and D-serine, or the lack of GDNF and IL-4. Activated microglia may increase the release of glutamate and D-serine and this may be attenuated by the SSRIs fluoxetine and citalopram, explaining the effects of these antidepressants on neuronal viability in co-culture. Another possible explanation is that activated microglia decrease their release profile of GDNF and IL-4, and the SSRIs fluoxetine and citalopram restore the released levels of these factors. The pro-inflammatory factors are unlikely candidates based on

the finding that the majority of the antidepressants decreased the release of TNF-α and NO from activated microglia, but only fluoxetine and citalopram attenuated the reduced viability of injured neurons in co-culture. If TNF-α and NO were indeed the key players, then the majority of the antidepressants tested should have attenuated this reduced viability; however, this was not the case. BDNF is another candidate that is highly unlikely to be a key player. In the brain, activated microglia increase the release of BDNF, but BDNF is a trophic factor that supports the growth and survival of neurons. In co-culture, activated microglia decrease the viability of injured neurons and it is counter-intuitive that an increase in the trophic BDNF leads to this effect on viability. Therefore, we did not expect BDNF to be one of the key players.

4.2. Materials and methods

4.2.1. Primary mixed glial cultures

Mixed glia were prepared from whole brains of post natal day 1 Sprague-Dawley rats. For detailed descriptions of the dissociation methods, refer to **Section 2.2.1.**

4.2.2. Primary microglial culture

After 21 DIV, microglia were isolated from mixed glial cultures via trypsinization (Saura et al. 2003). For a detailed description of the trypsinization method, refer to **Section 2.2.2.**

4.2.3. Primary cortical neurons

Cortical neurons were cultured from embryonic Sprague-Dawley day 18 rats based on previous methods (Brewer et al. 1993; Swayze et al. 2004; Lai and Todd 2006). For detailed descriptions of the dissociation methods, refer to **Section 3.2.3.**

4.2.4. Antidepressants/chemicals

Non-activated and LPS-activated microglia were pre-treated with three classes of antidepressants, namely TCAs (imipramine and clomipramine), MAOIs (phenelzine and TCP) and SSRIs (fluoxetine and citalopram). LPS (Sigma) was chosen as a microglial activator. For antagonists, the following compounds were used: MK-801 (NMDA receptor antagonist), L701,324 (glycine and D-serine site NMDA receptor antagonist), aminoguanidine (iNOS inhibitor, AG), anti-TNF-α antibody (TNF-α scavenger), and anti-BDNF antibody (BDNF scavenger). The NMDA receptor antagonist L701,324 (hereafter referred to as L701) was purchased from Sigma. For HPLC analysis, all amino acid standards (L-glutamine, glycine, D-serine, L-serine, GABA, taurine, L-glutamine, L-alanine, L-aspartate, and L-arginine) were purchased from Sigma.

4.2.5. Neuronal OGD insult and the assessment of cortical neuron viability

For detailed descriptions, refer to **Sections 3.2.5 and 3.2.6.**

4.2.6. L701 and MK-801 antagonism of the NMDA receptor *in vitro*

To our knowledge, no study has used L701 as an antagonist under *in vitro* experimental conditions. Thus to determine if L701 is an effective antagonist at the NMDA receptor glycine and D-serine site *in vitro*, neurons were injured using toxic levels of the amino

acids L-glutamate, glycine, and D-serine. Briefly, neurons were exposed to conditions that included: 5μM glutamate with or without 10 nM of L701 or 20 μM of MK-801; 5 μM glutamate and 5 μM glycine with or without 10 nM L701 or 20 μM of MK-801; 5 μM glutamate and 5 μM D-serine with or without 10 nM L701 or 20 μM of MK-801; and 10 nM of L701 or 20 μM of MK-801 alone. When L701 was used, the antagonist was added to the cortical neurons 30 min prior to glutamate, glycine, or D-serine addition. For L701, the concentration of 10 nM was chosen based on dose-response studies where the other concentrations tested (20 nM, 30 nM, and 40 nM) had no additional effect compared to 10 nM (data not shown). The concentration of MK-801 (20 μM) was determined from previous reports (King et al. 2007; Klimaviciusa et al. 2008) and was effective in antagonizing the NMDA receptor in neuronal cultures in our experiments. Following all manipulations, cultures were left to incubate for 24 hrs and neuronal viability was assessed with the MTT assay.

For the co-culture experiments, the antagonists L-701 and MK-801 were added to neurons 24 hrs after OGD insult and the cells were left to incubate for a further 30 min before transfering of inserts into microglial wells. The co-cultures were left to incubate for 24 hrs, and neuronal survival was then assessed.

4.2.7. TNF-α scavenging, BDNF scavenging, and iNOS inhibition in microglia

AG (2 μM), anti-TNF-α Ab (0.1 μg/ml), or anti-BDNF Ab (0.1 μg/ml) was added to microglia 30 min before LPS stimulation, and the mixtures were left to incubate for 24 hrs. AG concentrations were determined from initial dose-response trials and were

consistent with a previous report (Kita et al. 2002). The concentrations of anti-TNF-α and anti-BDNF Abs were determined from initial dose-response trials and were consistent with manufacturer guidelines for *in vitro* use. After incubation, microglial medium was removed and replaced with Neurobasal medium supplemented with AG, anti-TNF-α, or anti-BDNF antagonists. Neuronal inserts were insulted with OGD for 40 min. After 24 hrs incubation, the inserts were transferred to microglia in Neurobasal medium. The co-cultures were left to incubate for another 24 hrs and neuronal survival was then assessed.

4.2.8. High performance liquid chromatography (HPLC)

Amino acid release from microglia was determined using conditions previously described (Grant et al. 2006). In brief, a C_{18} column (Waters Symmetry, 3.5µm, 4.6 x 150mm) with a Waters guard column (5µm, 3.9 X 20mm) was used. Proteins were precipitated out of the medium using methanol (3:1 volume methanol:sample). The mixtures were vortexed thoroughly, kept on ice for 10-20 min, centrifuged for 5 min (10,000 x g, 4°C), and the supernatants collected. The derivatizing reagent (reacted for 5 min with samples just prior to HPLC analysis) consisted of 1mg o-phthaldialdehyde and 2mg N-isobutyryl-L-cystine in 0.1ml methanol and 0.9ml 0.1M sodium borate buffer at pH 9.5. Mobile phase A contained 8.40g NaH_2PO_4 (Fisher) and 1.42g Na_2HPO_4 (Fisher) dissolved in 1700ml distilled H_2O and 300ml methanol; pH was adjusted to 6.2 with 10M NaOH. Mobile phase B contained 6.43g NaH_2PO_4 dissolved in 1340mL distilled H_2O and 1130ml methanol; pH was adjusted to 6.2 with 10M NaOH and 60ml tetrahydrofuran (Fisher) was added. Both mobile phases were filtered through a 0.2µm membrane. The mobile

phase gradient began at 80% A: 20% B at a flow rate of 0.5 mL/min; the final mixture of mobile phase was 0% A and 100% B. The total runtime for each sample was 60 min, with all the compounds eluting by 40 min. The fluorescent derivatives were detected using a Shidmazu RF-10A detector, with excitation wavelength set at 344nm and emission wavelength set at 443nm. The sample chamber and column temperatures were controlled at 4°C and 30°C, respectively.

Amino acid stock solutions were prepared at a concentration of 1.0mg/mL in distilled H_2O and diluted to give 6-point standard curves with a blank for each amino acid. Levels of nine amino acids were analyzed: L-glutamate, L-serine, D-serine, glycine, L-glutamine, L-aspartate, taurine, L-alanine, and GABA. Other than L-glutamate and D-serine, there were no changes in amino acid levels between different experimental conditions. Amino acid peaks from samples were compared to those in the standard curves to determine amino acid levels using Empower2 software. Final values were calculated after protein normalization. For a detailed description of the BCA protein assay, refer to **Section 2.2.6**.

4.2.9. BDNF, GDNF, IL-4, and TNF-α ELISAs

Different ELISA protocols were used depending on the factor of interest. For a detailed description of the TNF-α ELISA protocol, refer to **Section 2.2.7.**

For IL-4, quantification was performed using an ELISA kit (R & D systems). The assays were conducted based on the protocols supplied by the manufacturer. Capture antibody

(2 μg/ml) was added to 96 well plates and left overnight. The wells were washed and blocked with 1% bovine albumin (BSA) in PBS for 1 hr. Samples and standards (100 μl) were added to the wells and left to incubate overnight at 4°C. After washing wells with PBS, 100 μl of biotinylated detection antibody (200 ng/ml) were added to the wells and left to incubate for 2 hrs at room temperature. After washing wells with PBS, 100 μl of HRP-conjugated streptavidin secondary antibody, diluted 1:200 from the stock, were added to the wells and left to incubate for 20 min at room temperature. After a final wash, a mixture of 30% hydrogen peroxide diluted in a solution of tetramethylbenzidine (TMB) (1:1000) was added to the wells, which were left to stand for approximately 20 min. The reaction was stopped by adding 1.8 N sulfuric acid to the wells and the absorbance was read at 450 nm using a plate reader. Plates were also read at 570 nm to account for background and these values were subtracted from the 450 nm readings. Values were normalized to protein levels. For a detailed description of the BCA protein assay, refer to **Section 2.2.6**.

For BDNF and GDNF ELISA, a competitive assay was used instead of an ELISA kit. Briefly, goat anti-BDNF or anti-GDNF antibody was diluted (1:100) in a coating buffer containing 100 mM sodium carbonate and 100 mM sodium bicarbonate (pH 9.6). This buffer was then mixed (1:1) with standards and samples in 96 well plates and incubated overnight at 4°C. Plates were washed 3x with PBS and incubated with blocking buffer (1% BSA in 1X PBS) for 2 hrs. Plates were washed another 3x and the anti-goat HRP-conjugated secondary antibody diluted in blocking buffer (1:2000) was added to the plated and incubated at room temperature for 30 min. After a final wash, a mixture of

30% hydrogen peroxide diluted in a solution of TMB (1:1000) was added to the wells, which were then left to stand for approximately 20 min. The reaction was stopped by adding 1.8 N sulfuric acid to the wells and the absorbance was read at 450 nm using a plate reader. Plates were also read at 570 nm to account for background and these values were subtracted from the 450 nm readings. Values were normalized to protein levels. For a detailed description of the BCA protein assay, refer to **Section 2.2.6**.

4.2.10. Statistical Analysis

Significance levels for data sets were calculated using a two-tailed t-test (designated with *) or a two way ANOVA followed by Bonferroni post hoc test (designated with #). t-Tests were used to compare two data sets involving the manipulation of only one variable. Two way ANOVAs were used to compare two data sets involving manipulations of two or more variables. Both designations * and # denote $p < 0.05$. Refer to figure legends for total 'n' values for specific experiments. For all MTT absorbance values, each 'n' represents one insert of a neuronal culture, and inserts were taken from at least three separate culture preparations for each study.

4.3. Results

4.3.1. Fluoxetine and citalopram attenuate the release of glutamate and D-serine from LPS-activated microglia

The first step in the studies reported in this chapter was to determine if microglia up-regulated the release of amino acids following LPS activation and if the antidepressants used in this study could attenuate any increases observed. Of the ten amino acids

measured, we found that LPS significantly increased the release of glutamate and D-serine from microglia compared to non-activated controls (**Figure 4-1 A and B**). Fluoxetine and citalopram attenuated this increase in glutamate and D-serine release from LPS-activated microglia, whereas the remaining antidepressants (imipramine, clomipramine, phenelzine, and TCP) failed to attenuate this increase (**Figure 4-1 A and B**). The antidepressant drugs did not affect the release of glutamate or D-serine from non-activated microglia compared to non-treated controls. Furthermore, we measured the release of other amino acids from non-activated and activated microglia including L-alanine, L-aspartate, glycine, L-glutamine, GABA, taurine, L-serine, and L-arginine (**data not shown**) and found that there were no differences in the release of these amino acids before or after activation. Following 24 hr treatment with the antidepressants, only phenelzine showed increases in GABA release from non-activated and activated microglia compared to non-treated control, but this increase was not significant (**data not shown**). The remaining antidepressants had no effect on the release of amino acids measured in this study from non-activated or activated microglia.

4.3.2. The release of trophic and anti-inflammatory factors from microglia following LPS-activation and antidepressant treatments

The next step was to determine if microglia up-regulated the release of the trophic factors, BDNF and GDNF, or the anti-inflammatory cytokine IL-4 following LPS activation and if the antidepressants used in this study could attenuate any increases observed. When we measured BDNF release, we found that LPS-activation increased the BDNF release from microglia compared to non-activated controls and that the pre-

treatment with the antidepressants failed to significantly attenuate this increase (**Figure 4-2 A**). In addition, we found that the treatment of microglia with the antidepressants imipramine, clomipramine, and fluoxetine increased the release of BDNF in non-activated microglia (**Figure 4-2 A**). When we measured the release of GDNF or IL-4, we found no differences in the release of these factors from LPS-activated microglia compared to non-activated controls; furthermore, the pre-treatment of non-activated or LPS-activated microglia with antidepressants also showed no differences in the release of GDNF or IL-4 (**Figure 4-2 B and C**).

4.3.3. Antagonists used in this study were effective *in vitro*

Next, we investigated which microglial-derived factors were the key players in mediating the viability of injured corticial neurons in the co-culture setup. Prior to carrying out this experiment, we selected various reported antagonists of a number of factors and performed experiments to verify that they are effective antagonists *in vitro.* The first antagonist selected was AG, an iNOS inhibitor that attenuates the production of NO in activated microglia. When we pre-treated microglia with 2, 3, or 4 μM of AG prior to LPS-activation, nitrite release was significantly decreased compared to non-treated activated controls (**Figure 4-3 A**). The pre-treatment with 1 μM AG also decreased this nitrite release from microglia, but this decrease was not significant. Therefore, an AG concentration of 2 μM was chosen to inhibit the microglial production of NO in co-culture.

In order to neutralize the activity of TNF-α and BDNF, we selected an anti-TNF-α and an anti-BDNF antibody, which scavenge TNF-α and BDNF, respectively, once these factors are released from microglia into the medium. We found that the pre-treatment with 0.05, 0.1, 0.2, or 0.5 μg/ml anti-TNF-α or anti-BDNF antibody prior to the activation of microglia significantly decreased the levels in free-floating TNF-α or BDNF in medium compared to activated controls (**Figure 4-3 B and C**). Therefore, antibody concentrations of 0.1 μg/ml were chosen to neutralize the microglial release of TNF-α and BDNF since it was the lowest concentration effective in attenuating levels of these factors to baseline.

To antagonize the effects of glutamate and D-serine released from microglia, we selected MK-801 and L701, two NMDA receptor antagonists. To test the effectiveness of these antagonists *in vitro*, cortical neurons were pre-treated with the antagonists and injured with excess L-glutamate, glycine, and D-serine to induce excitotoxicity. We found that the addition of 5 μM of L-glutamate to cortical neurons significantly reduced viability compared to healthy controls (**Figure 4-4 A**). The co-administration of 5 μM glycine with L-glutamate to neurons further decreased this viability, which was even further decreased when 5 μM D-serine was co-administered with L-glutamate (**Figure 4-4 A)**. The pre-treatment of cortical neurons with the MK-801 antagonist (20 μM) prior to any of the toxic treatments completely attenuated these decreases in viability (**Figure 4-4 B).** The pre-treatment of cortical neurons with the L701 antagonist (10nM) significantly attenuated the decreases in viability following the co-administration of L-glutamate with glycine or D-serine, but did not effect viability following the administration of L-

glutamate alone (**Figure 4-4 B**). The treatments of glycine or D-serine without L-glutamate had no effect on the viability of neurons compared to healthy controls (**Figure 4-4 A**). MK-801 and L701 concentrations of 20 μM and 10 nM were chosen to antagonize the NMDA receptors on the neurons in co-culture.

4.3.4. The scavenging of TNF-α release from LPS-activated microglia significantly attenuates the losses in neuronal viability in co-culture

To investigate if the microglial release of NO or TNF-α was important in causing the reduction in the viability of OGD-injured neurons in co-culture, LPS-activated microglia were pre-treated with 0.1 μg/ml of anti-TNF-α antibody or 2 μM of AG for 24 hrs before co-culture. After 24 hrs in co-culture, we found that activated microglia pre-treated with the TNF-α scavenger significantly attenuated this decrease in viability; however, viability was still significantly less than the viability of injured neurons prior to co-culture (**Figure 4-5**). On the other hand, activated microglia pre-treated with AG did not attenuate the loss in viability seen in co-cultures with non-treated activated microglia (**Figure 4-5**). The pre-treatment of activated microglia with both the TNF-α scavenger and AG showed no differences when compared to the pre-treatment with the TNF-α scavenger alone, showing that there was no combination effect (**Figure 4-5**). These results show that the neutralization of TNF-α release can restore some viability lost following the exposure of injured neurons to activated microglia in co-culture, whereas the inhibition of NO production does not have this effect.

4.3.5. The antagonism of the NMDA receptor on cortical neurons significantly attenuates the losses in neuronal viability in co-culture

To investigate if the microglial release of glutamate or D-serine were important in causing the reduction in the viability of OGD-injured neurons in co-culture, we treated the OGD-injured neurons with 20 μM of MK-801 or 10 nM of L701 for 30 min prior to co-culture. After 24 hrs in co-culture, we found that injured neurons treated with MK-801 showed complete attenuation of reduced viability, returning it back to the levels observed prior to co-culture with activated microglia (**Figure 4-6**). In addition, we found that injured neurons treated with L-701 also showed attenuation in reduced viability, but this attenuation was not as complete as observed with MK-801 (**Figure 4-6**). The treatment of injured neurons by both MK-801 and L701 showed no differences when compared to the treatment of the MK-801 alone, showing that there was no combination effect (**Figure 4-6**). These results show that the antagonism of the NMDA receptor with MK-801 can fully restore the viability lost following the exposure of injured neurons to activated microglia in co-culture, whereas L701 only partially restores this loss in viability.

4.3.6. The scavenging of BDNF released from SSRI-treated LPS-activated microglia has no effect on the viability of injured neurons following co-culture

Finally, we investigated if BDNF release was a key player in causing the attenuation in the loss in viability of OGD-injured neurons in co-culture with activated microglia. We showed that fluoxetine attenuated the loss in viability of injured neurons in co-cultures with activated microglia (**Figure 3-5**) and increased BDNF release from microglia

(**Figure 4-2 A**). Therefore, we suggested that fluoxetine might increase BDNF release from microglia to support neuronal viability when injured neurons are exposed to activated microglia. To test this, SSRI-treated activated microglia were pre-treated with 0.1 μg/ml of anti-BDNF antibody for 24 hrs to scavenge released BDNF. If BDNF release from microglia following SSRI treatments was a key factor in attenuating the decrease in the viability of injured neurons, then the scavenging of BDNF would abolish this attenuation. We found that after 24 hrs in co-culture, the anti-BDNF pre-treatment of SSRI-treated activated microglia showed no differences in the viability of neurons compared to the SSRI-only treatment groups (**Figure 4-7**). The scavenging of BDNF did not affect the viability of neurons in co-cultures with fluoxetine- or citalopram-treated activated microglia; therefore, an increase in BDNF release from microglia is not a key player in attenuating the loss in viability following co-culture.

4.4. Summary

In this chapter, we determined the key players that are released from activated microglia and decrease the viability of OGD-injured neurons in co-culture. We were skeptical that NO and TNF-α were key players based on antidepressant effects on the release of these factors from microglia and antidepressant effects on the viability of injured neurons in co-culture, where these two findings did not correlate. The studies described in this chapter were devoted to finding the key players released from activated microglia and investigating which antidepressants can attenuate this release.

We measured various factors in the medium from non-activated and LPS-activated microglial cultures. We found increases in the levels of the amino acids glutamate and D-serine and the trophic factor BDNF released from LPS-activated microglia. We also measured eight additional amino acids, the trophic factor GDNF, and the anti-inflammatory cytokine IL-4, and the release of none of these was increased from LPS-activated microglia. When we pre-treated these activated microglia with antidepressants, we found that fluoxetine and citalopram were the only antidepressants tested that attenuated the release of glutamate and D-serine from activated microglia, correlating with the results of the effects of these antidepressants on neuronal viability in co-culture. Therefore, we were optimistic that glutamate and D-serine were the key players.

To demonstrate that glutamate and D-serine are these key players, we applied various antagonists in the co-cultures. The antagonism of NO and BDNF release did not show any effects on the viability of injured neurons after co-cultures with activated microglia; however, the antagonism of the NMDA receptors on neurons and TNF-α release from microglia attenuated this decrease in viability. Therefore, these antagonism studies suggest that glutamate, D-serine, and TNF-α release from activated microglia are the key players.

This chapter has revealed some of these key players, but the mechanisms behind antidepressant actions on attenuating the release of glutamate and D-serine from activated microglia has yet to be elucidated. In the next chapter, we investigate the mechanisms

behind the effects of fluoxetine and citalopram in attenuating glutamate and D-serine release.

4.5. Figures

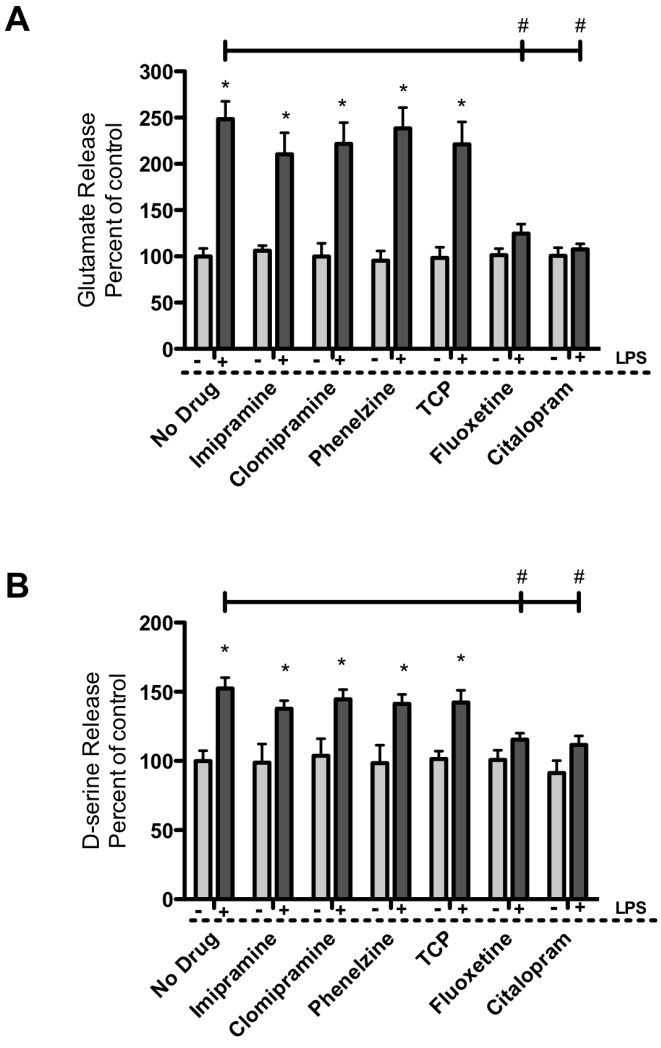

Figure 4-1 - Glutamate and D-serine release by non-activated and activated microglia following antidepressant treatments. Cells were treated with or without 1 μg/ml LPS 30 min following 10 μM antidepressant treatment. **A.** LPS significantly induced microglial release of glutamate into the medium compared to non-activated controls; fluoxetine and citalopram significantly decreased LPS-induced glutamate release into medium, whereas the other antidepressants had no effect, n = 8. **B.** LPS significantly induced microglial release of D-serine into the medium compared to non-activated controls; fluoxetine and citalopram significantly decreased LPS-induced D-serine release into the medium, whereas the other antidepressants had no effect, n = 8. Antidepressants had no effect on the release of glutamate or D-serine from non-stimulated microglia. * < 0.05 (comparisons within the same drug group), #<0.05 (comparisons against LPS-activated untreated microglia).

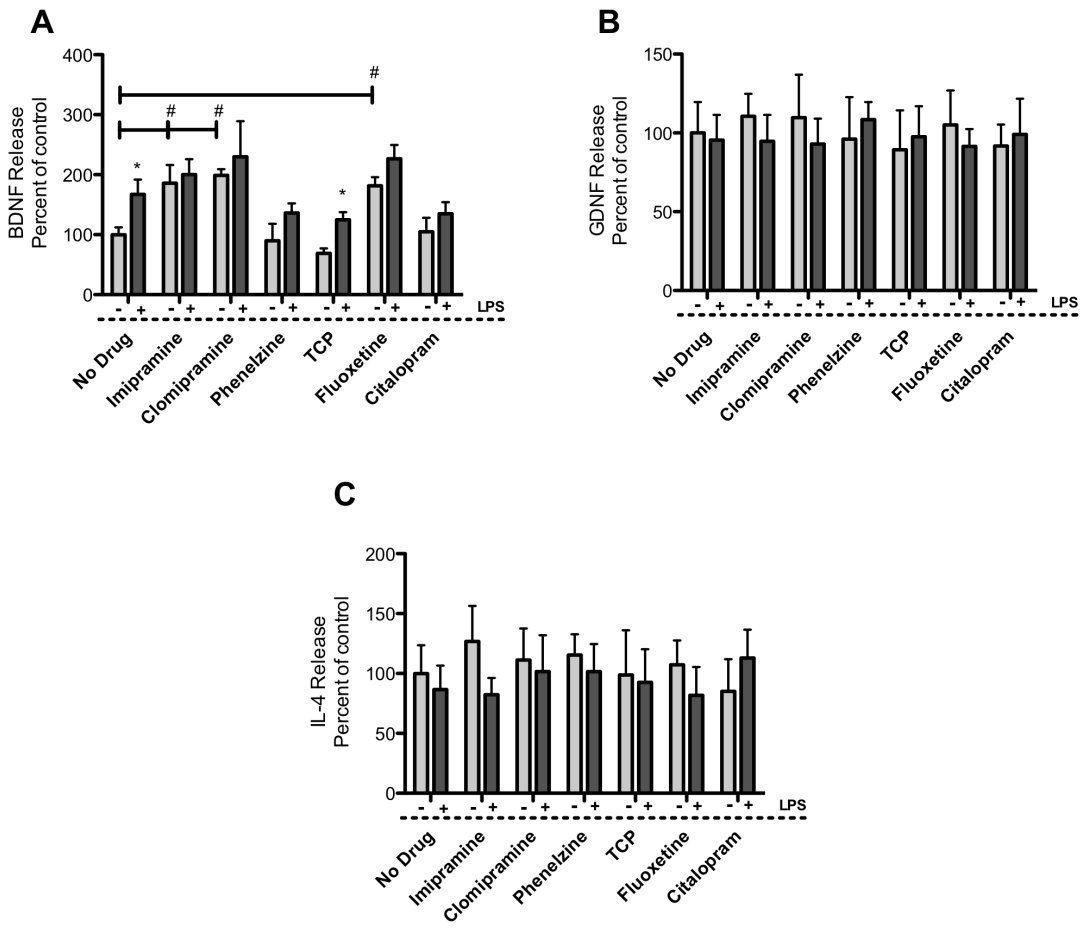

Figure 4-2 – BDNF, GDNF, and IL-4 release by non-activated and activated microglia following antidepressant treatments. Cells were treated with or without 1 μg/ml LPS 30 min following 10 μM antidepressant treatments. **A.** LPS significantly induced microglial release of BDNF into the medium compared to non-activated controls; none of the antidepressants tested significantly decreased this release. Imipramine, clomipramine, and fluoxetine induced BDNF release from non-activated microglia. n = 10. **B.** Neither LPS or the antidepressants affected the release of GDNF from non-activated or activated microglia. n = 5. **C.** Neither LPS or the antidepressants effected the release of IL-4 from non-activated or activated microglia. n = 5. [* represents $p < 0.05$ (comparisons within the same drug group) and # represents $p < 0.05$ (comparisons against non-activated untreated microglia)].

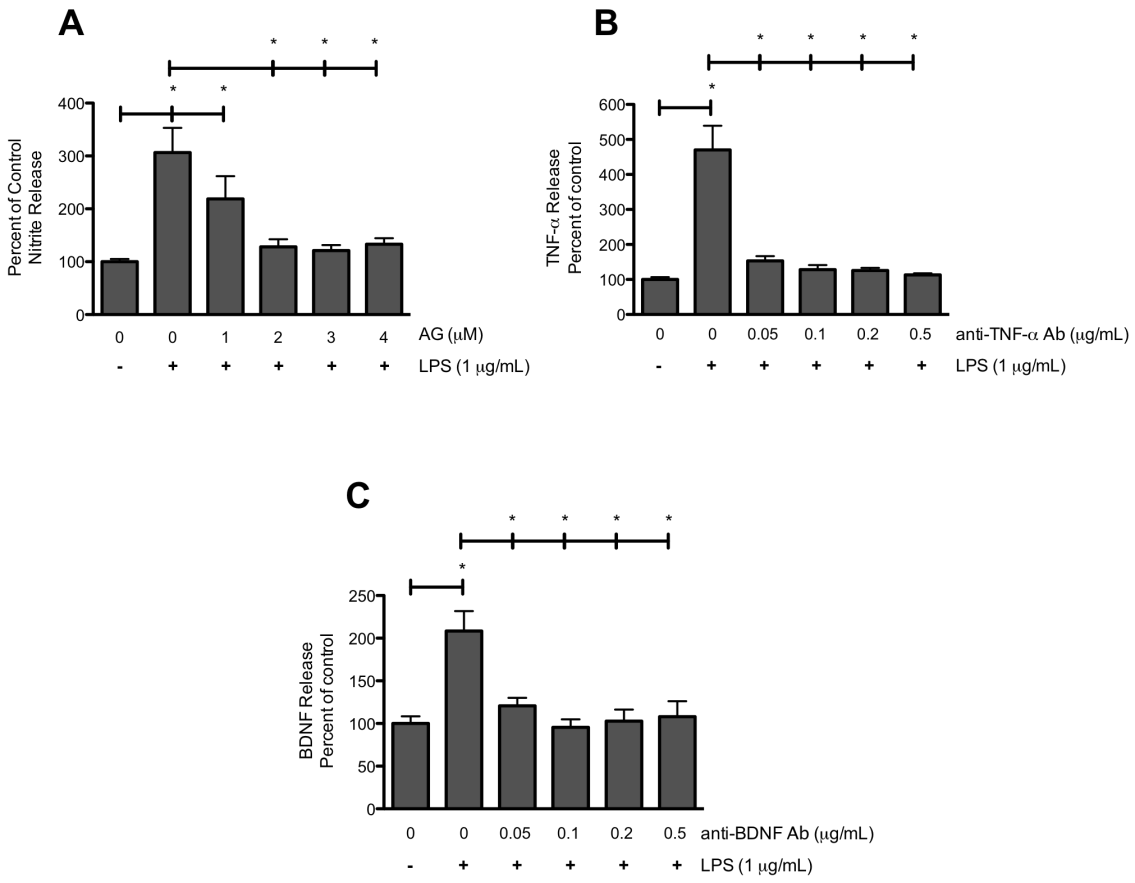

Figure 4-3 – Histograms displaying the effectiveness of an iNOS inhibitor and scavengers for TNF-α and BDNF *in vitro*. A. Aminoguanidine (AG) at concentrations 2 μM, 3 μM, and 4 μM attenuated the release of nitrite from activated microglia, wheras AG at 1 μM caused a small decrease that was not significant, n = 8. **B.** All tested concentrations of the anti-TNF-α antibody (Ab) scavenged the release of TNF-α from activated microglia, n = 8. **C.** All tested concentrations of the anti-BDNF antibody (Ab) scavenged the release of BDNF from activated microglia, n = 8. [* represents $p < 0.05$].

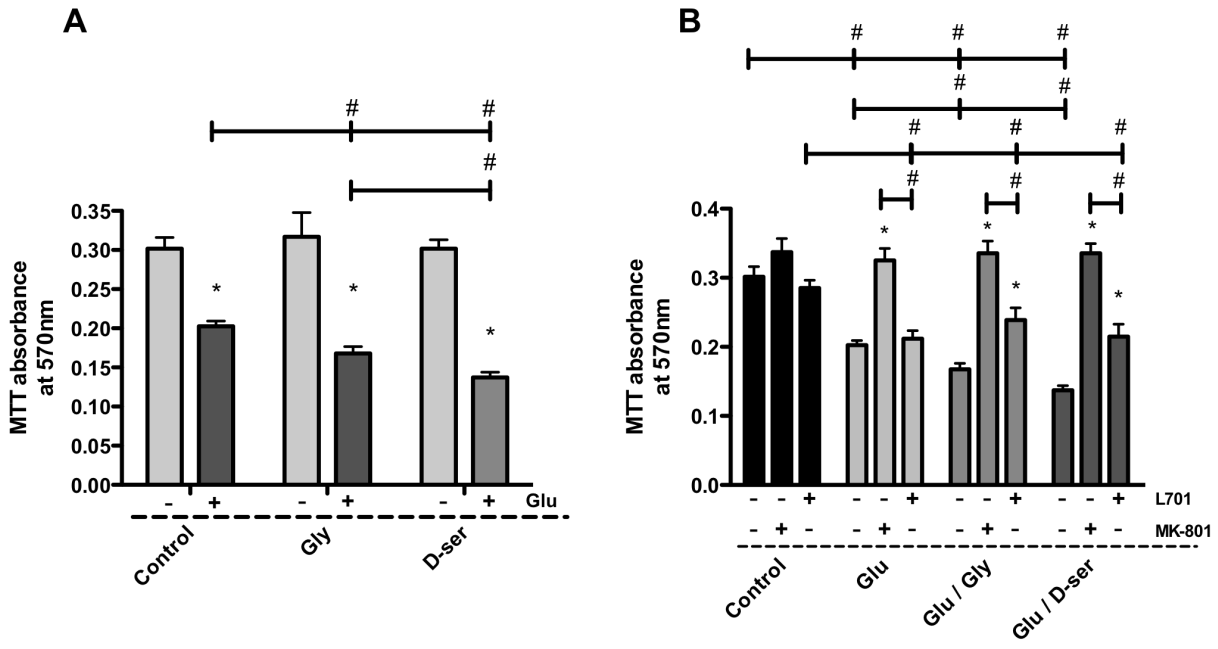

Figure 4-4 – Histograms displaying the effectiveness of the NMDA antagonists MK-801 and L701 *in vitro*. **A**. L-glutamate (Glu, 5 μM) significantly decreased neuronal viability compared to untreated controls after 24 hrs of culture; addition of 5 μM glycine (Gly) with 5 μM Glu further decreased this viability; addition of 5 μM D-serine (D-Ser) with 5 μM Glu decreased this viability even further, n = 8. **B.** Treatments of healthy neurons with MK-801 or L701 had no effect on neuronal viability compared to untreated controls. Pre-treatment of neurons given 5uM Glu with L701 did not attenuate this loss in viability; pre-treatment of neurons given 5 μM Glu and 5 μM Gly or 5 μM D-Ser with L701 significantly attenuated this loss in viability compared to Glu/Gly or Glu/D-Ser toxic conditions; however, this increase in viability did not reach control levels. Pre-treatment with MK-801 in all toxic conditons attenuated the loss in viability back to control levels, n = 8. [* represents $p < 0.05$ (comparisons between groups with one manipulated variable) and # represents $p < 0.05$ (comparisons between groups with two or more manipulated variables)].

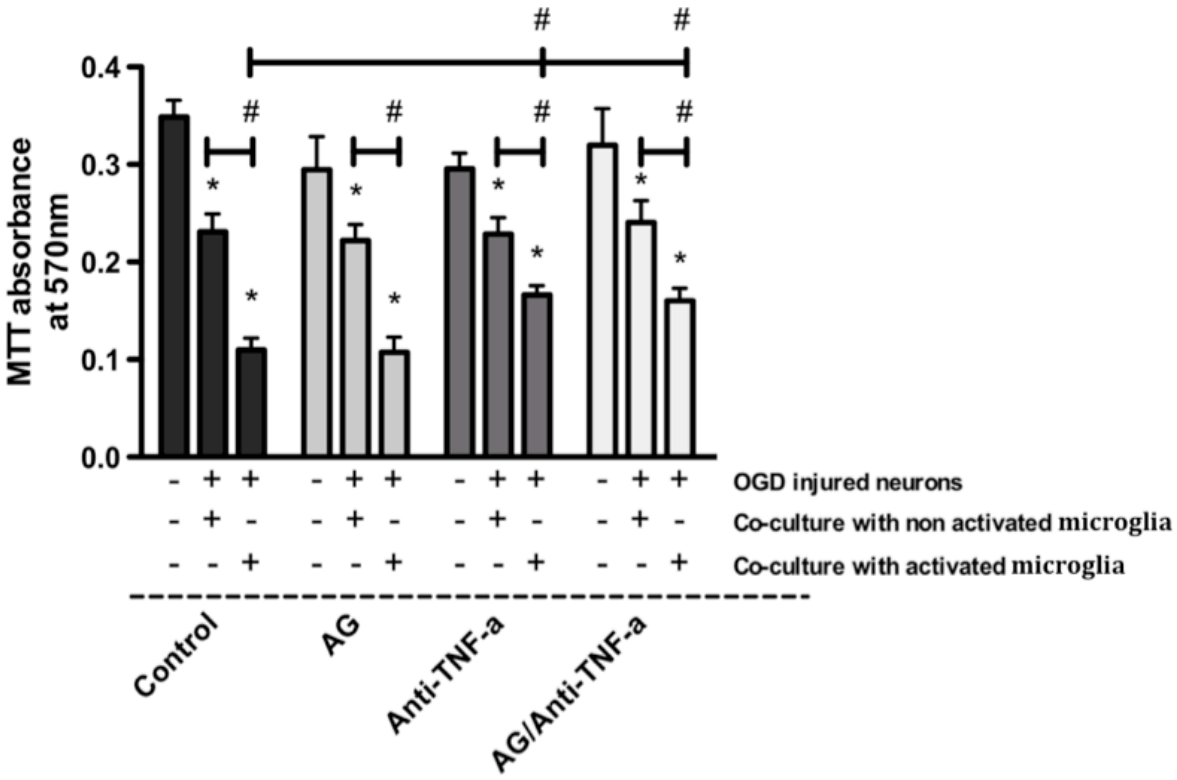

Figure 4-5 – Pre-treatment of non-activated and activated microglia with aminoguanidine (AG) and the TNF-α scavenger (anti-TNF-α antibody) and their effects on the viability of injured neurons after 24 hrs co-culture. Neither AG nor anti-TNF-α Ab had an effect on neuronal viability when added directly on healthy neurons. The pre-treatment of non-activated microglia with AG or the anti-TNF-α Ab 24 hrs prior to co-culture did not effect the viability of OGD-injured neurons. Viability of OGD-injured neurons was further decreased when neurons were placed in co-cultures with pre-activated microglia; however, this loss in viability was significantly attenuated by the pre-treament of activated microglia with anti-TNF-α Ab, whereas AG did not have this effect. The co-administration of both AG and anti-TNF-α Ab was not significantly different compared to the administration of only anti-TNF-α Ab. [* represents $p < 0.05$ (comparisons between groups with one manipulated variable) and # represents $p < 0.05$ (comparisons between groups with two or more manipulated variables)].

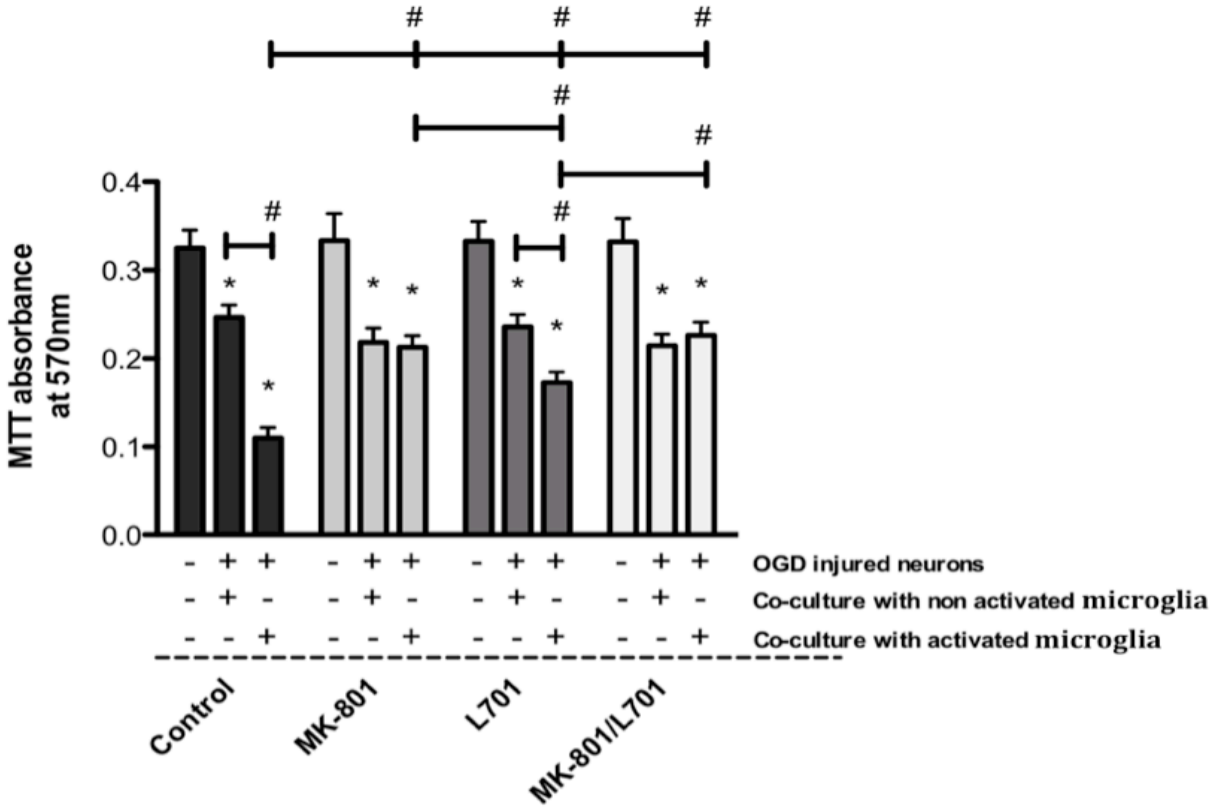

Figure 4-6 – Treatment of cortical neurons following OGD injury with MK-801 and L701 and their effects on the viability of injured neurons after 24 hrs co-culture with non-activated and activated microglia. Neither MK-801 nor L-701 had an effect on neuronal viability when added directly to healthy neurons. OGD-injury significantly decreased the viability of cortical neurons and this viability was further decreased following co-culture with activated microglia; the treatment of OGD-injured neurons with MK-801 or L701 prior to co-culture significantly attenuated this further decrease in viability. MK-801 attenuated this further decrease in viability significantly more compared to L701. The co-administration of both MK-801 and L701 did not differ in attenuating this further decrease in viability compared to the administration of only MK-801; however, it was significant compared to L701 alone. [* represents $p < 0.05$ (comparisons between groups with one manipulated variable) and # represents $p < 0.05$ (comparisons between groups with two or more manipulated variables)].

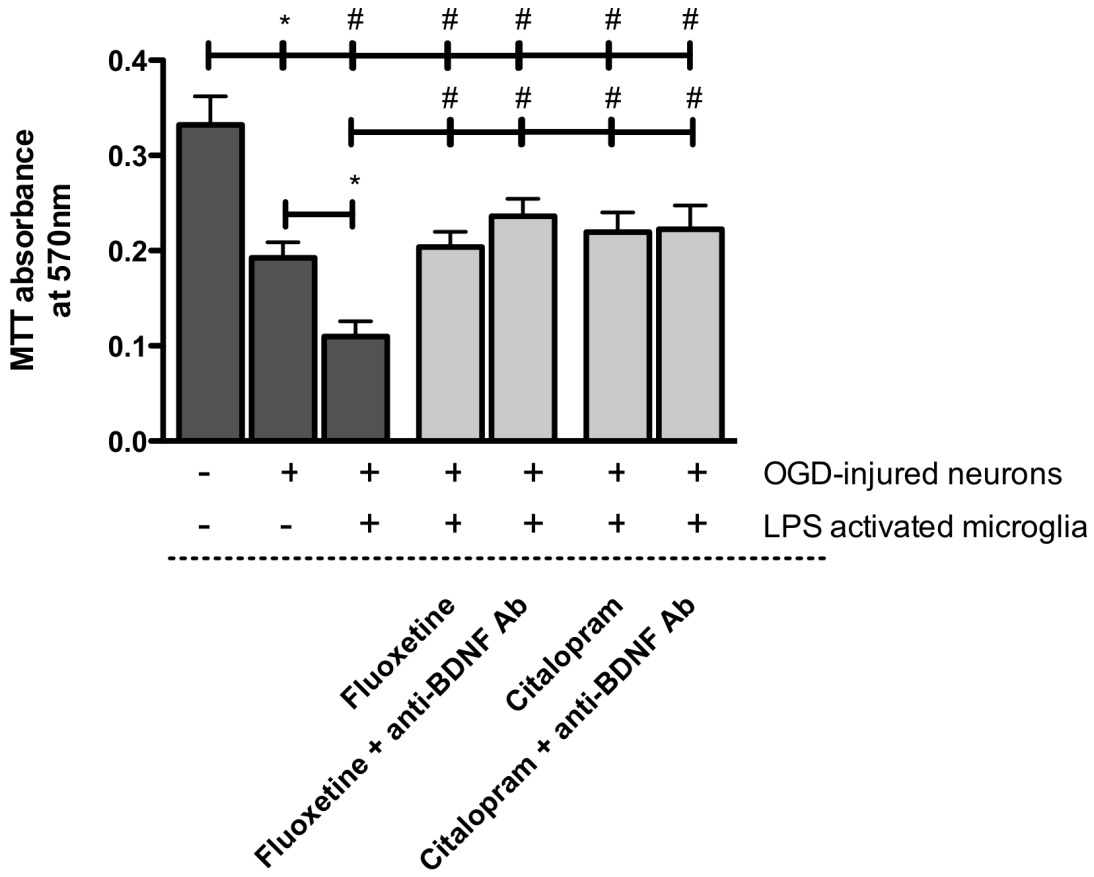

Figure 4-7 – Pre-treatment of activated microglia with fluoxetine or citalopram and the BDNF scavenger (anti-BDNF antibody) and its effects on the viability of injured neurons after 24 hrs co-culture. OGD significantly decreased neuronal viability compared to healthy controls and this viability was further decreased following 24 hrs of co-culture with activated microglia. The pre-treatment with fluoxetine or citalopram significantly attenuated this further decrease in viability and the co-administration of anti-BDNF antibody with these SSRIs had no effect on this attenuation. [* represents $p < 0.05$ (comparisons between groups with one manipulated variable) and # represents $p < 0.05$ (comparisons between groups with two more manipulated variables)].

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CHAPTER 5

Antidepressant effects on the induction of microglial apoptosis

5.1. Introduction

5.1.1. Antidepressant effects on intracellular signaling cascades in microglia

Chapter 4 showed that the key players in reducing the viability of injured neurons when exposed to LPS-activated microglia in co-culture were glutamate, D-serine, and to a lesser extent, TNF-α. As discussed in chapter 3, fluoxetine and citalopram were the sole antidepressants tested that attenuated the release of glutamate and D-serine from LPS-activated microglia. However, the mechanisms behind these SSRI effects in attenuating glutamate and D-serine release have yet to be investigated.

There are various studies that have investigated the anti-inflammatory properties of antidepressants on activated microglia. The majority of these studies have focused on the intracellular signaling cascades involving the MAP kinases and NF-κB. For instance, Hwang et al (2008) showed that clomipramine and imipramine inhibited IκB degradation, nuclear translocation of the p65 subunit of NF-κB, and phosphorylation of p38 MAP-kinase in LPS-activated microglia. In addition, studies have shown that fluoxetine inhibits the microglial NF-κB signaling pathway following LPS-activation (Liu et al. 2011; Zhang et al. 2012). Despite the fact that various antidepressants attenuate these intracellular cascades, it is still uncertain where the exact site of action is for these antidepressants. One possibility is that antidepressants may bind to a receptor on the cell surface of microglia, initiating a cascade that interferes with the MAP kinase and NF-κB intracellular signaling cascades following activation. Another possibility is that antidepressants can bypass the cell membrane of microglia and interfere directly with the phosphorylation of kinases although there is a lack of evidence behind this claim.

However, in the studies described in this chapter, we discovered that fluoxetine can induce microglial apoptosis, thereby deregulating these intracellular signaling cascades following LPS-activation.

5.1.2. Apoptosis

An understanding of the mechanisms behind the process of apoptosis in mammalian cells have come in large part from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (Horvitz 1999). Apoptosis has been recognized and accepted as a unique and essential mode of "programmed" cell death, a genetically determined elimination of cells. Apoptosis is necessary during development and occurs normally during aging and is a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson 2001).

Observations under light microscopy have shown that apoptotic cells are smaller in size with a dense cytoplasm and organize tightly packed organelles in compartments called apoptotic bodies (Kerr et al. 1972). Apoptosis also involves pyknosis, characterized by nuclear chromatin condensation. The organelle integrity is still maintained and enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed primarily by macrophages and there is essentially no or little inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding

interstitial tissue and (2) they are quickly phagocytosed by surrounding cells (Savill and Fadok 2000; Kurosaka et al. 2003).

5.1.3. Caspases

Caspases are proteases that are expressed in an inactive form in most cells and once activated can activate other downstream caspases, initiating a cascade. This cascade amplifies the apoptotic signaling pathway and thus leads to rapid cell death. Activated caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids. Caspases have been broadly categorized into initiators (caspase-2,-8,-9,-10) and effectors (caspase-3,-6,-7) (Cohen 1997; Rai et al. 2005). Other caspases have been identified (caspase-1,-4,-5,-11,-12,-13,-14) and been implicated in apoptotic processes following inflammation, amyloid-β toxicity, and embryonic development (Hu et al. 1998; Nakagawa et al. 2000; Kang et al. 2002).

5.1.4. Extrinsic vs. intrinsic pathways of apoptosis

The mechanisms of apoptosis are highly complex and involve energy-dependent cascades. To date, research indicates that there are two main apoptotic pathways: the extrinsic pathway and the intrinsic pathway. However, there is evidence that these pathways can influence each other (Igney and Krammer 2002). Nonetheless, both the extrinsic and intrinsic pathways converge to cleave caspase-3 and this results in DNA fragmentation, degradation of nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors, and finally uptake

by phagocytic cells (Hengartner 2000). The extrinsic apoptotic signaling pathway involves death receptors that are members of the TNF receptor gene superfamily (Locksley et al. 2001). Members of the TNF receptor family have a cytoplasmic domain of about 80 amino acids called the death domain and this domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways (Ashkenazi and Dixit 1998). The best-characterized ligands and corresponding death receptors include Fas/FasR and TNF-α/TNFR1 (Ashkenazi and Dixit 1998). Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to the Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to the TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP (Hsu et al. 1995; Wajant 2002). Caspase 8 then binds to the death domain and becomes activated by FADD, and then translocates through the cytoplasm to activate other caspases (Kischkel et al. 1995).

The intrinsic apoptotic signaling pathways involve stimuli that produce intracellular signals that are mitochondrial-initiated rather than receptor-mediated. These stimuli cause changes in the inner mitochondrial membrane that result in an opening of pores in the mitochondrial membrane, a loss of the mitochondrial membrane potential, and the release of sequestered pro-apoptotic proteins such as cytochrome c from the inter-membrane space into the cytosol (Du et al. 2000; Saelens et al. 2004). Once in the cytosol, cytochrome c binds and activates the apoptotic protease activating factor-1 (Apaf-1) as well as caspase-9, forming the apoptosome and leading to caspase-9

activation (Chinnaiyan 1999; Hill et al. 2004). A second group of pro-apoptotic proteins, AIF and endonuclease G, are released from the mitochondria during apoptosis, but this is a late event that occurs after the cell has committed to dying. AIF and endonuclease G translocate to the nucleus and cause DNA fragmentation and condensation of nuclear chromatin and both function in a caspase-independent manner (Joza et al. 2001; Li et al. 2001). Regulation of the release of apoptotic proteins from the mitochondria occurs through members of the Bcl-2 family of proteins (Cory and Adams 2002) which governs mitochondrial membrane permeability and can be either pro-apoptotic (Bcl-10, Bax, Bak, Bid, Bad) or anti- apoptotic (Bcl-2, Bcl-x, Bcl-XL). These proteins have a special significance since they can determine if the cell commits to apoptosis or aborts the process. The regulation of cytochrome c release from the mitochondria is the consequence of the actions of the Bcl-2 family of proteins and their effects on mitochondrial membrane permeability.

Serine phosphorylation of Bad is often associated with multifunctional phosphoserine binding molecules, and when Bad is phosphorylated it is trapped by these binding molecules and sequestered in the cytosol; however, when Bad is unphosphorylated, it translocates to the mitochondria to release cytochrome c (Zha et al. 1996). In addition, Bad can form complexes with the anti-apoptotic proteins Bcl-Xl or Bcl-2 to neutralize their protective effects (Yang et al., 1995). Both Bcl-2 and Bcl- Xl inhibit the release of cytochrome c from the mitochondria but fail to when sequestered by Bad.

The extrinsic and intrinsic pathways both lead to the cleavage and activation of caspase-

3. Activated caspase-3 cleaves various substrates including poly (ADP-ribose) polymerase (PARP), plasma membrane proteins, and nuclear proteins and ultimately causes the morphological and biochemical changes seen in apoptotic cells (Slee et al. 2001). Caspase-3 is activated by various caspases (caspase-8, -9, or -10) and induces disintegration of the cell into apoptotic bodies.

5.1.5. Chapter objectives

In the studies described in this chapter, we investigated whether fluoxetine disrupts the release of glutamate, D-serine, and inflammatory factors by inducing apoptosis of microglia during activation. The idea that fluoxetine may promote microglial apoptosis comes from observed decreases in the overall protein levels in the cell lysates of fluoxetine-treated microglial cultures. We measured microglial viability, protein levels of cell lysates, caspase-3 and caspase-8 expression, and immuno-fluorescent images showing fluoxetine's effects on the induction of apoptosis in microglia. We also compared these effects to those of other antidepressants, including imipramine, phenelzine, and citalopram. Since we have shown that fluoxetine and citalopram are the only antidepressants tested to attenuate glutamate and D-serine release from LPS-activated microglia, we hypothesized that these SSRIs exert this effect by inducing microglial apoptosis. We did not expect imipramine and phenelzine to induce microglial apoptosis since they were unable to attenuate glutamate and D-serine release from LPS-activated microglia.

5.2. Materials and methods

5.2.1. Primary mixed glial cultures

Mixed glia were prepared from whole brains of post natal day 1 Sprague-Dawley rats. For detailed descriptions of the dissociation methods, refer to **Section 2.2.1.**

5.2.2. Primary microglial culture

After 21 DIV, microglia were isolated from mixed glial cultures via trypsinization (Saura et al. 2003). For a detailed description of the trypsinization method, refer to **Section 2.2.2.**

5.2.3. Antidepressants/chemicals

Three classes of antidepressants were used in pretreatment of non-activated and LPS-activated microglia prior to co-culture: TCAs (imipramine), MAOIs (phenelzine), and SSRIs (fluoxetine, citalopram, and norfluoxetine). Staurosporine (STS, Sigma) was used as a positive inducer of apoptosis. TNF-α (R and D systems) was used as a positive inducer of the extrinsic apoptotic pathway. The concentration of TNF-α (20 ng/ml) was chosen based on previous literature (Boldin et al. 1996; Fernandes-Alnemri et al. 1996; Muzio et al. 1996).

5.2.4. Assessment of microglial viability and protein levels

Microglial viability was assessed using the MTT colorimetric assay. Briefly, 100µl of 5 mg/ml MTT were added to microglial wells and left to incubate for 40 min at 37°C. Medium was aspirated and replaced with 210µl of dimethyl sulfoxide (DMSO) for cell

lysis. Absorbance was read at 570nm on a plate reader (Powerwave X, Bio-Tek). For a detailed description of the protein assay, refer to **Section 2.2.6.**

5.2.5. Immunocytochemistry

Microglia were cultured in 12-well plates. Cells were fixed with 10% formalin for 10 min. After 3 washes with PBS, the cells were incubated with a blocking solution [10% normal horse serum (Gibco) in PBS with 0.1% Triton X-100] for 1hr. After washing, cells were incubated and co-labeled with Iba-1 (1:1000, Wako) or cleaved-caspase 3 (1:500, Cell signaling) primary antibodies (diluted in PBS with 1% normal horse serum) overnight at 4°C. After 3 additional washes, wells were incubated with the relevant fluorescent-conjugated secondary antibodies (1:2000, Invitrogen) diluted in PBS for 1hr. After a final wash, wells were cover-slipped using aqueous mounting medium. Lieca imaging software coupled to an inverted fluorescent microscope was used to capture images of Iba1 and cleaved-caspase 3 labeled microglia. Nine images were captured per well for each filter. Merged images were created by Image-J software.

5.2.6. Nitrite assay

For a detailed description of the nitrite assay, refer to **Section 2.2.6.**

5.2.7. Enzyme-linked immunosorbant assay (ELISA)

For a detailed description of TNF-α measurements with ELISA, refer to **Section 2.2.7.**

5.2.8. Live/dead staining of microglia

Staining of live or dead microglia before and after antidepressant treatments was performed using specific fluorescent-conjugated nucleic acid stains in 12-well plates. Following 24 hrs of antidepressant treatment, medium was removed and wells were washed three times. Fresh DMEM/F-12 medium was added to the wells. To each well, a final concentration of 5 μM of SYTO green-fluorescent nucleic acid stain (Molecular Probes) was added and the plates were incubated at 37°C at 5 % CO_2 for 110 min. The green nucleic acid stain is permeable to all cells and stains both live and dead cells. Plates were then removed and a final concentration of 0.1 μM of SYTOX orange nucleic acid stain (Molecular Probes) was added to the wells and plates were incubated at 37°C at 5 % CO_2 for 10 min. The SYTOX orange stain is non-permeable to healthy cells and only stains compromised or dead cells. The medium was then removed, and wells were washed two times with PBS and cover-slipped using aqueous mounting medium. Lieca imaging software coupled to an inverted fluorescent microscope was used to capture images of these wells. Nine images were captured per well for each filter. Merged images were created by Image-J software. The green stain was visualized at a wavelength of 488 nm and SYTOX orange was visualized at a wavelength of 550 nm.

5.2.9. Western Blotting (WB)

WB was used to determine cleaved-caspase 3 (CC3), caspase 3 (C3), cleaved-caspase 8 (CC8), caspase 8 (C8) and β-actin expression in non-treated and antidepressant-treated microglial cell lysates. Briefly, 9 to 12 microglial wells (in 12-well plates) were pooled and lysed with TEEN buffer (0.8% Triton X-100 and 0.2% SDS) after 24 hrs of antidepressant treatment. Gels (15% acrylamide) were loaded with 15-22 μg of protein

per well. The gels were run for 2 hrs under a voltage of 120 volts using BioRad western blotting equipment in running buffer. Gels were then placed in transfer buffer and proteins were transferred to PVDF membrane (Millipore) and left at 4°C overnight at 0.08 amps. The following day, the membranes were washed with wash buffer, and blocked for 1 hr with 10% skim milk powder (diluted in wash buffer) at room temperature on a shaker. After two 10 min washes on a shaker, the primary antibodies, rabbit anti-rat CC3 (1:1000, Cell Signaling), rabbit anti-rat C3 (1:1000, Cell Signaling), rabbit anti-rat CC8 (1:1000, Cell Signaling), rabbit anti-rat C8 (1:1000, Cell Signaling), or mouse anti-rat β-actin (1:5000) were added to the membranes and then mixtures were left overnight on a shaker at 4°C. Primary antibodies were diluted in 1% bovine serum and 0.02% sodium azide in wash buffer, and re-used multiple times (up to 5) to save antibody. After three 10 min washes, membranes were incubated with appropriate HRP-conjugated 2° antibodies (diluted in 5 % skim milk solution) for 1 hr at room temperature. After another three 10 min washes, enhanced chemiluminescence (ECL, Amersham) was added to membranes for 4 min and the mixtures then placed in a film cassette. The membranes were exposed to Kodak film for varying amounts of time and then the films were developed using a Kodak processor.

5.2.10. Gas Chromatography-Mass Spectrometry (GC-MS)

Fluoxetine and norfluoxetine levels in medium were measured by GC-MS using a modification of an assay described previously in our laboratory (Rotzinger et al. 1997). Standards were prepared in naive culture medium. Cell medium from fluoxetine-, norfluoxetine-, or non-treated microglia (450 μl) was used in the analytical procedure.

Fluvoxamine (100 ng) was added as internal standard to the samples, which were basified (to pH 8.5) by the addition of 50 μl potassium bicarbonate. A solution of 2 ml toluene and 2 μl pentafluorobenzoyl chloride (PFBC) was added to each sample tube. The tubes were capped and shaken vigorously for 15 min and centrifuged at 1800 *g* for 5 min in a benchtop centrifuge. The organic phase was pipetted into 100mm x 13 mm screw cap culture tubes and taken to dryness in a Savant evaporator (Speed Vac SC 110, Fisher Scientific). Toluene (100 μl) was added to each tube and the tubes were vortexed well. The toluene was transferred to a silanized glass insert and 2 μl was injected onto an Agilant 6890 GC coupled to a 5973n MS with an electron ionzation (EI) source. Data were collected in the single ion monitoring (SIM) mode. The column used was HP-5MS (5% phenyl methyl siloxane; 30m x 0.25 m, 0.25 μM), with a gas flow rate of 1 ml/min helium.

5.2.11. Statistical Analysis

Significance levels for data sets were calculated using a two-tailed t-test (designated with *) or a two way ANOVA followed by Bonferroni post hoc test (designated with #). t-Tests were used to compare two data sets involving the manipulation of only one variable. Two way ANOVAs were used to compare two data sets involving manipulations of two or more variables. * and # denote $p < 0.05$. Refer to figure legends for total 'n' values for specific experiments. For all NO and ELISA experiments, each 'n' represents one set of cultures with a replicate of three wells per condition.

5.3. Results

5.3.1. Fluoxetine decreases the viability of microglia and the protein levels in cell lysates

In previous experiments described in this thesis, we have shown that fluoxetine and citalopram were the only antidepressants of the six tested that attenuated glutamate and D-serine release from LPS-activated microglia; therefore, we investigated the possible mechanisms behind this result. Upon close examination of previous data, we found that fluoxetine (10 μM) significantly attenuated the protein levels from microglial cell lysates compared to non-treated controls and that higher doses of fluoxetine (30 and 40 μM) lowered these protein levels even further (**Figure 5-1 A**). From this result, we hypothesized that a possible mechanism behind the attenuation of glutamate and D-serine release from activated microglia is by the induction of microglial apoptosis after fluoxetine or citalopram treatment. If these antidepressants are inducing apoptosis of microglia, these compromised cells may fail to release the toxic factors upon activation. The next step was to measure microglial viability with the MTT assay, and we found that the addition of fluoxetine at any of the doses tested, excluding 5 μM, decreased the viability of microglia compared to non-treated controls (**Figure 5-1 B**). Similar to what was found with protein levels, higher doses of fluoxetine (30 and 40 μM) further decreased MTT viability compared to the fluoxetine dose of 10 μM. When examining the morphology of fluoxetine-treated microglia under a phase-contrast microscope, we observed that those cells treated with fluoxetine (10, 20, 30, and 40 μM) showed increases in the number of spherical-like cells compared to non-treated controls, which displayed more elongated cells and few spherical cells (**Figure 5-1 C**). The

morphological transformation of microglia from elongated or ramified to spherical is one classic hallmark of apoptosis.

5.3.2. Phenelzine, imipramine, and citalopram do not decrease the viability of microglia and the protein levels in cell lysates

Next we investigated if phenelzine, imipramine, or citalopram affected the viability of microglia. We did not expect phenelzine and imipramine to have an effect on this viability since these antidepressants did not attenuate the release of glutamate and D-serine from LPS-activated microglia, whereas we did expect citalopram to have an effect since it attenuates the release of these factors. As expected, phenelzine (**Figure 5-2 A**) and imipramine (**Figure 5-2 B**) did not decrease the viability of microglia at any dose tested; however, treatments of 5 μM phenelzine slightly increased microglial viability compared to non-treated controls (**Figure 5-2 A**). Interestingly, citalopram did not effect the viability of microglia at any dose tested compared to non-treated controls, suggesting that citalopram does not have the same effects on microglial viability as fluoxetine (**Figure 5-2 C**).

5.3.3. Fluoxetine, but not citalopram, phenelzine, or imipramine, increases cleaved-caspase 3 expression and decreases caspase 3 expression in microglia

To further investigate if fluoxetine and citalopram induced apoptosis of microglia, we measured expression of cleaved-caspase 3, a protease essential in apoptosis. Apoptosis involves the cleavage of the protease caspase 3 within the cytoplasm to produce cleaved-caspase 3; therefore, apoptotic cells show decreases in caspase 3 and increases in

cleaved-caspase 3 levels. Western blot analysis showed that fluoxetine significantly increased cleaved-caspase 3 expression, whereas caspase 3 expression levels were decreased compared to non-treated controls (**Figure 5-3 A, Figure 5-3 C, Figure 5-3 D**). Citalopram (**Figure 5-3 B, Figure 5-3 C, Figure 5-3 D)**, imipramine, or phenelzine had no significant effect on cleaved-caspase 3 or caspase 3 expression levels compared to non-treated controls (**Figure 5-4 A, Figure 5-4 B, Figure 5-4 C**). These results suggest that fluoxetine induces apoptosis of microglia, whereas citalopram, phenelzine, and imipramine do not.

We immuno-labeled antidepressant-treated microglia against Iba1 and cleaved-caspase 3 to further display the increase in apoptotic activity in microglia following fluoxetine treatment. Compared to non-treated controls (**Figure 5-5 A**), the fluoxetine-treated group (10 μM) significantly displayed a greater number of microglia co-labeled with Iba1 and cleaved-caspase 3 (**Figure 5-5 B**). Photomicrographs taken at a higher magnification (40X objective) clearly show microglial cells co-labeled for Iba1 and cleaved-caspase 3 (**Figure 5-5 C**). The treatment of microglia with 10 μM of citalopram (**Figure 5-5 E**), imipramine (**Figure 5-5 F**), or phenelzine (**Figure 5-5 G**) showed no differences in co-labeled cells compared to non-treated controls (**Figure 5-5 D**). The combination of western blot and immuno-fluorescent data clearly shows that fluoxetine increases the microglial expression of cleaved-caspase 3, a hallmark of apoptosis.

5.3.4. Fluoxetine, but not citalopram, phenelzine, or imipramine, increases the number of compromised microglial cells and reduces the percentage of live cells

The next step was to investigate if the increase in cleaved-caspase 3 after fluoxetine treatment led to microglial death. To test this, we used a live and dead staining protocol to distinguish healthy intact cells from compromised damaged cells. Two stains were used: STYO green, a stain that penetrates all live or dead cells, and SYTOX orange, a stain that penetrates only those cells with compromised cellular membranes. A compromised or dying cell will co-label for both the green and orange stains, whereas healthy cells will label only with the green stain and not take up any orange stain. We found that the number of co-stained cells (dead cells) in the imipramine (**Figure 5-6 B**), phenelzine (**Figure 5-6 C**), or citalopram (**Figure 5-6 E**) groups did not differ compared to non-treated controls (**Figure 5-6 A, Figure 5-6 D**). For a positive control to induce apoptosis, we treated microglia with 100 nM of staurosporine. After the treatment with fluoxetine (**Figure 5-6 F**) or staurosporine (**Figure 5-6 G**), we found a significant increase in the percentage of co-stained cells compared to non-treated controls (**Figure 5-6 A, Figure 5-6 D**). Fluoxetine induces cell death in addition to increasing the expression of cleaved-caspase 3 in microglia, whereas the other antidepressants tested did not have these effects.

5.3.5. Norfluoxetine attenuates the release of pro-inflammatory factors from activated microglia and reduces microglial viability

In a human subject, fluoxetine is primarily metabolized in the liver to form norfluoxetine, a SSRI with as strong a potency at inhibiting 5-HT reuptake and a much longer elimination half-life than its parent fluoxetine. Since norfluoxetine is highly prevalent in the depressed brain following fluoxetine treatment, we investigated if norfluoxetine had

similar effects as fluoxetine in attenuating pro-inflammatory factors released from microglia and reducing their cell viability. We found that norfluoxetine significantly attenuated the release of NO and TNF-α from LPS-activated microglia (**Figure 5-7 A, Figure 5-7 B**). In addition, norfluoxetine decreased the viability of microglia at the doses of 10, 20, and 30 μM compared to non-treated controls (**Figure 5-7 C**). Finally, norfluoxetine decreased the protein levels within microglial cell lysates at 10, 20, and 30 μM compared to non-treated controls, with 20 and 30 μM doses having the greatest effect (**Figure 5-7 D**). Norfluoxetine attenuates the release of NO and TNF-α from activated microglia and decreases the viability of these cells, similar to what was observed with fluoxetine.

5.3.6. Norfluoxetine increases cleaved-caspase 3 expression and decreases caspase 3 expression in microglia

We investigated if norfluoxetine also induces the expression of cleaved-caspase 3 similarly to fluoxetine. Norfluoxetine at 10 and 20 μM increased the expression of cleaved-caspase 3 and decreased the expression of caspase 3 compared to non-treated controls (**Figure 5-8 A, Figure 5-8 B, Figure 5-8 C**). Norfluoxetine did not reach the marked effects of staurosporine (a positive inducer of apoptosis) in either the increase in cleaved-caspase 3 or the decrease in caspase 3 expressions in microglia (**Figure 5-8 B, Figure 5-8 C**). When we measured the number of microglial cells co-labeled against Iba1 and cleaved-caspase 3, we found that norfluoxetine (**Figure 5-9 B**) significantly increased the percentage of co-labeled cells compared to non-treated controls (**Figure 5-9 A, Figure 5-9 D**). As expected, staurosporine also increased the percentage of these co-

labeled microglial cells and these percentages were greater than in the norfluoxetine-treated groups (**Figure 5-9 C, Figure 5-9 D**). However, norfluoxetine increases cleaved-caspase 3 and decreases caspase 3 expression in microglia to a similar degree as fluoxetine.

5.3.7. Norfluoxetine increases the number of compromised microglial cells and reduces the number of live cells

We investigated if norfluoxetine also increased the percentage of dead cells similarly to fluoxetine; therefore, we co-stained norfluoxetine-treated microglia with SYTO green and SYTOX orange. Norfluoxetine (**Figure 5-10 B**) significantly increased the percentage of co-stained (dead) cells compared to non-treated controls (**Figure 5-10 A, Figure 5-10 D**). As expected, staurosporine also increased the percentage of these co-stained (dead) microglial cells and these percentages were greater than in the norfluoxetine-treated groups (**Figure 5-10 C, Figure 5-10 D**). Norfluoxetine increased the percentage of dead microglial cells similarly to fluoxetine.

5.3.8. Microglia do not metabolize fluoxetine to form norfluoxetine

We questioned whether microglia were able to convert fluoxetine to norfluoxetine, and if norfluoxetine was the main determinant in inducing microglial apoptosis and death. To answer this question, we measured the levels of fluoxetine and norfluoxetine in the medium of fluoxetine-treated microglia with GC-MS. If microglia treated with fluoxetine converted the drug into norfluoxetine, then we would measure a significant amount of norfluoxetine within the medium compared to non-treated controls. When we

ran fluoxetine-treated medium through the GC-MS 24 hrs post treatment, we found that there were insignificant levels of norfluoxetine in the medium, with 0.09% conversion taking place (**Figure 5-11**). As expected, a huge spike for fluoxetine in microglial medium was observed after fluoxetine treatment (**Figure 5-11**). Microglia do not convert fluoxetine to norfluoxetine, but both of these SSRIs have similar effects in decreasing microglial viability. MS confirmed the structures of fluoxetine and norfluoxetine by observing the fragmentation of both compounds.

5.3.9. Fluoxetine may induce apoptosis through an extrinsic pathway

The final step in this chapter was to investigate if fluoxetine and norfluoxetine induced apoptosis via the intrinsic or extrinsic pathway. We measured the expression levels of cleaved-caspase 8 and caspase 8 in fluoxetine- or norfluoxetine-treated microglia to determine if the extrinsic pathway in apoptosis was activated. Western blots revealed that fluoxetine and staurosporine decreased the expression of caspase 8 in microglia compared to non-treated controls (**Figure 5-12 A, Figure 5-12 D**). As a positive control, microglia were treated with TNF-α to induce the extrinsic pathway of apoptosis. TNF-α decreased the expression of caspase 8 but norfluoxetine failed to cause this decrease in microglial caspase 8 levels (**Figure 5-12 A, Figure 5-12 D**). When we examined cleaved-caspase 8 levels, western blots revealed no positive bands for any of the conditions, suggesting something faulty with the primary antibody (**Figure 5-12 A**). A decrease in caspase 8 should yield an increase in cleaved-caspase 8 levels, but this was not the case. Cleaved-caspase 3 levels were increased in fluoxetine-, norfluoxetine-, and staurosporine-treated groups compared to non-treated controls, but fluoxetine and

staurosporine showed significantly greater levels than norfluoxetine (**Figure 5-12 A, Figure 5-12 B**). Finally, caspase 3 levels were decreased in TNF-α-, fluoxetine-, norfluoxetine-, and staurosporine-treated groups compared to non-treated controls, but fluoxetine and staurosporine showed significantly lower levels than norfluoxetine (**Figure 5-12 A, Figure 5-12 C**). Although there is evidence that fluoxetine decreases caspase 8 expression, we cannot conclude that the extrinsic pathway of apoptosis is activated in microglia after fluoxetine treatment since cleaved-caspase 8 expression could not be measured.

5.4. Summary

In this chapter, we investigated a possible mechanism involved in the attenuation of glutamate and D-serine from LPS-activated microglia following fluoxetine and citalopram treatment. Upon closer inspection of data from fluoxetine-treated microglia, we found that fluoxetine decreased the protein levels measured from cell lysates. In addition, microglia displayed reduced viability and apoptotic morphology following fluoxetine treatment. Therefore, we hypothesized that fluoxetine and citalopram induce the apoptosis of microglia and this could be a possible mechanism in the attenuation of glutamate and D-serine from LPS-activated microglia.

Western blotting analysis highlighted increases in the apoptotic protease cleaved-caspase 3 in fluoxetine-treated microglia, an observation that was verified with immuno-fluorescent co-labeling, whereas these cells display decreases in caspase 3. In addition, co-staining with live and dead stains show that fluoxetine-treated microglial groups have

a greater percentage of dead cells compared to controls. The other antidepressants tested, including imipramine and phenelzine, showed no changes in cleaved-caspase 3 or caspase 3 expression in microglia or the percentage in dead cells. This result was expected since imipramine or phenelzine did not attenuate glutamate or D-serine release from LPS-activated microglia. However, citalopram-treated cells yielded unexpected results. Citalopram did not change the expression levels in cleaved-caspase 3 or caspase 3 in microglia or the percentage of dead cells. Therefore, the mechanism behind the attenuation in glutamate and D-serine release from microglia following citalopram treatment is likely not due to apoptosis.

The final step in the study described in this chapter was to determine if fluoxetine induced the extrinsic pathway of apoptosis in microglia. We found that fluoxetine decreased the expression of caspase 8 in microglia, suggesting that this extrinsic pathway is induced. However, we failed to measure expression levels of cleaved-caspase 8 and could not confirm the involvement of the extrinsic pathway. Therefore, fluoxetine induces apoptosis of microglia, but the exact pathway still needs to be elucidated. This induction of apoptosis may be the cause of the reduced release in glutamate and D-serine from activated microglia in the presence of this drug.

5.5 Figures

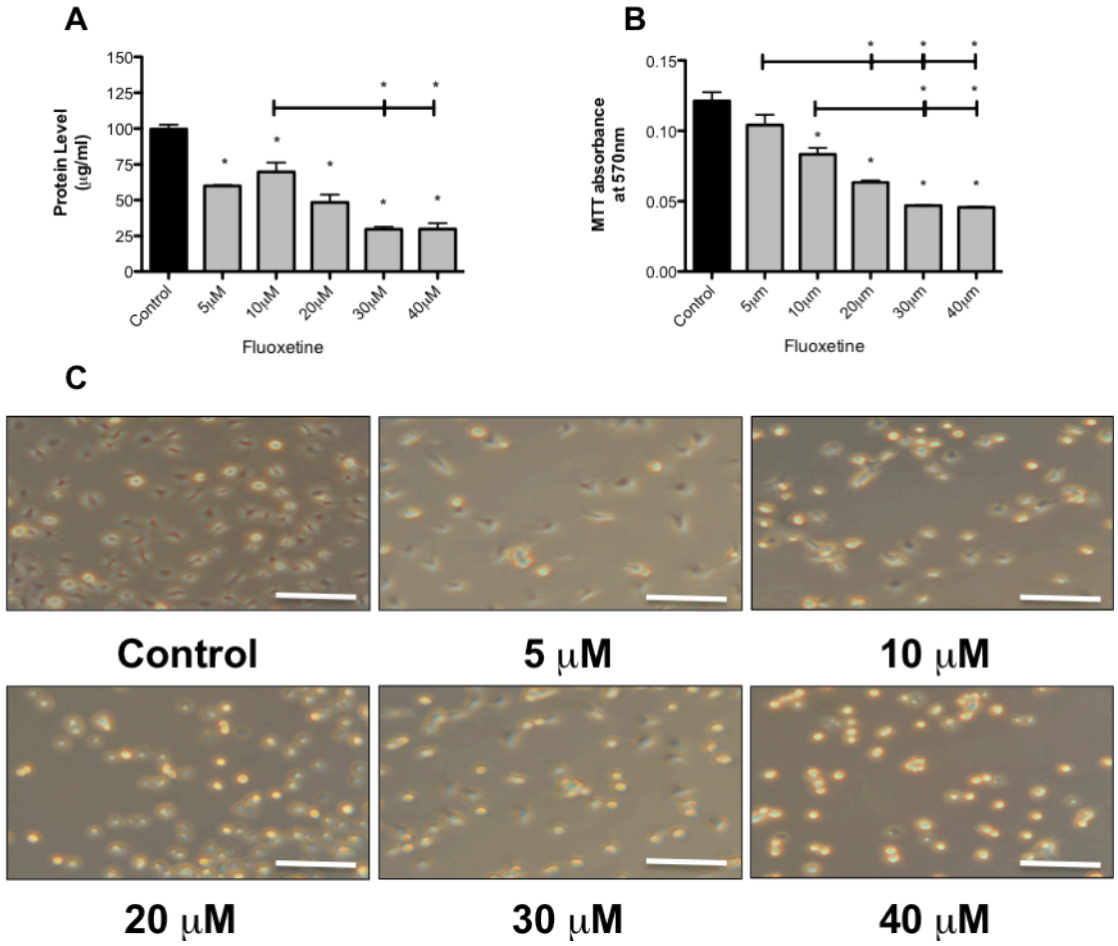


Figure 5-1 – Fluoxetine decreases the protein levels in microglial cell lysates and decreases microglial viability in a concentration-dependent manner. A. All fluoxetine treated microglial cells showed a decrease in the protein levels in cell lysates compared to non-treated controls. Microglia treated with 30 and 40 µM fluoxetine showed a greater reduction in protein levels compared to 5 or 10 µM treatments, n = 10. **B.** All fluoxetine-treated microglial cells, excluding 5 µM treatments, showed a decrease in MTT absorbance (viability) compared to non-treated controls. Microglia treated with 30 and 40 µM fluoxetine showed a greater reduction in viability compared to 5 or 10 µM treatments n = 10. **C.** Phase-contrast photomicrographs of microglia showing morphological changes following fluoxetine treatments. Fluoxetine-treated microglia acquire a spherical apoptotic-like morphology compared to control cells and this effect is concentration-dependent. Scale bar = 80 µM.

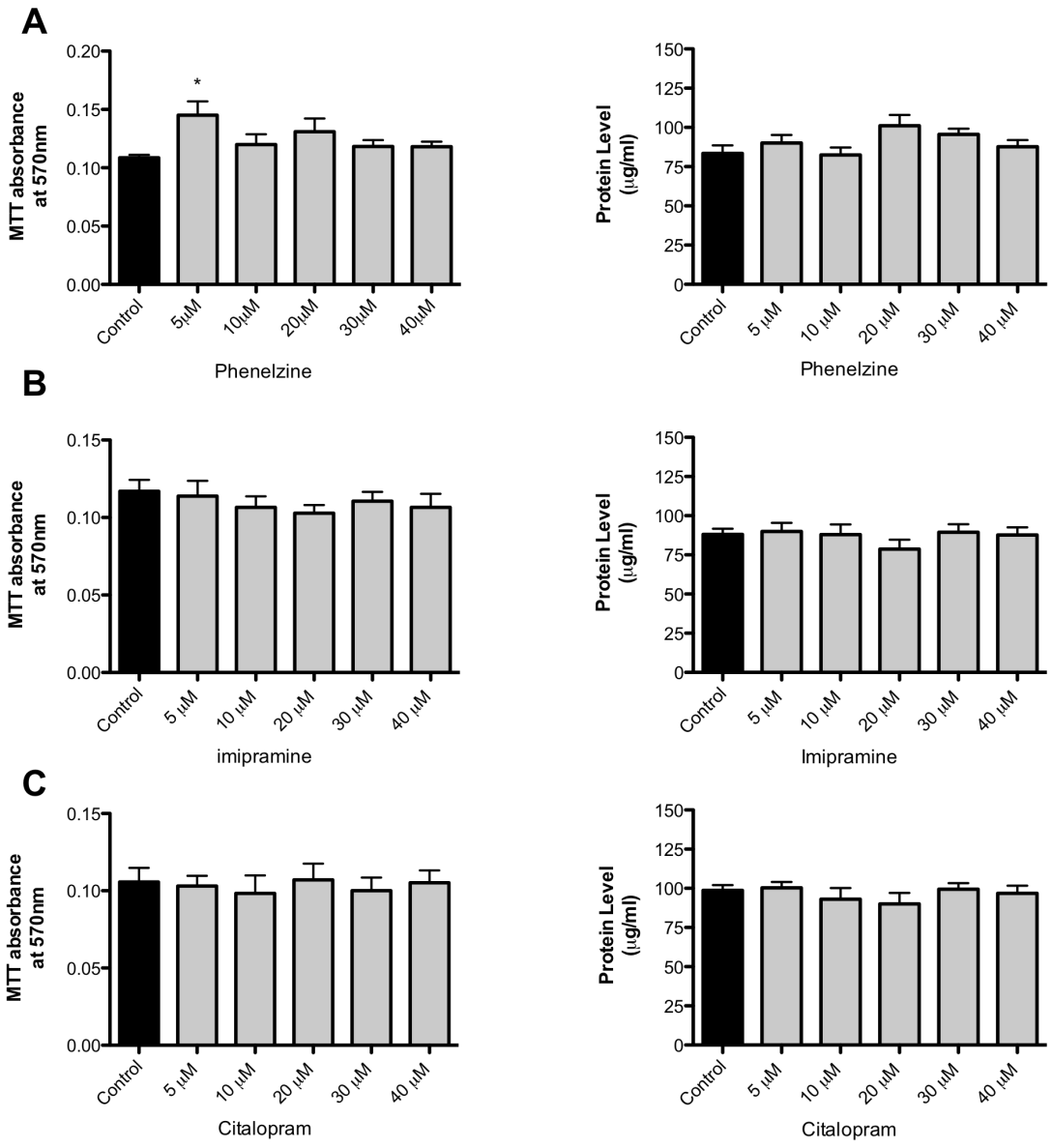


Figure 5-2 – The effect of phenelzine, imipramine, and citalopram on protein levels in microglial cell lysates and on microglial viability. A. All phenelzine concentrations tested, excluding 5 μM, showed no differences in the protein levels in cell lysates or cell viability compared to non-treated controls, whereas phenelzine at 5 μM increased microglial viability, n = 8. **B.** All imipramine concentrations tested showed no differences in the protein levels in cell lysates or cell viability compared to non-treated controls, n = 8. **C.** All citalopram concentrations tested showed no differences in the protein levels in cell lysates or cell viability compared to non-treated controls n=8. Statistical significance, * < 0.05 (compared to control).

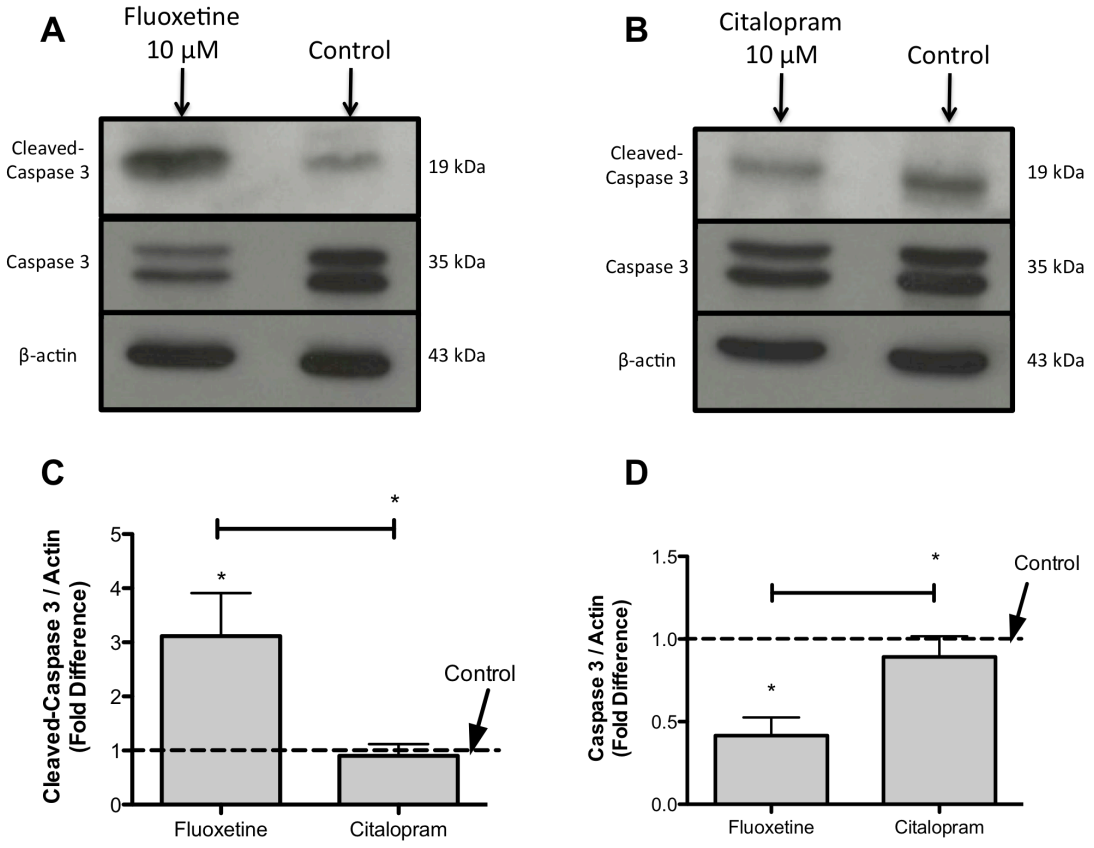


Figure 5-3 – Western Blot analysis for cleaved-caspase 3 and caspase 3 expression in microglial cell lysates following fluoxetine and citalopram treatment. A. Fluoxetine increases the expression of cleaved-caspase 3 (19 kDa) and decreases caspase 3 (35 kDa) expression compared to non-treated controls. **B.** Citalopram does not alter the expression levels of cleaved-caspase 3 or caspase 3 compared to non-treated controls. β-actin (43 kDa) expression was measured as a loading control. **C and D.** Histograms displaying cleaved caspase 3 (**C**) and caspase 3 (**D**) expression from western blot analysis, n = 5. Statistical significance, * < 0.05 (comparisons to control and comparisons between drug groups).

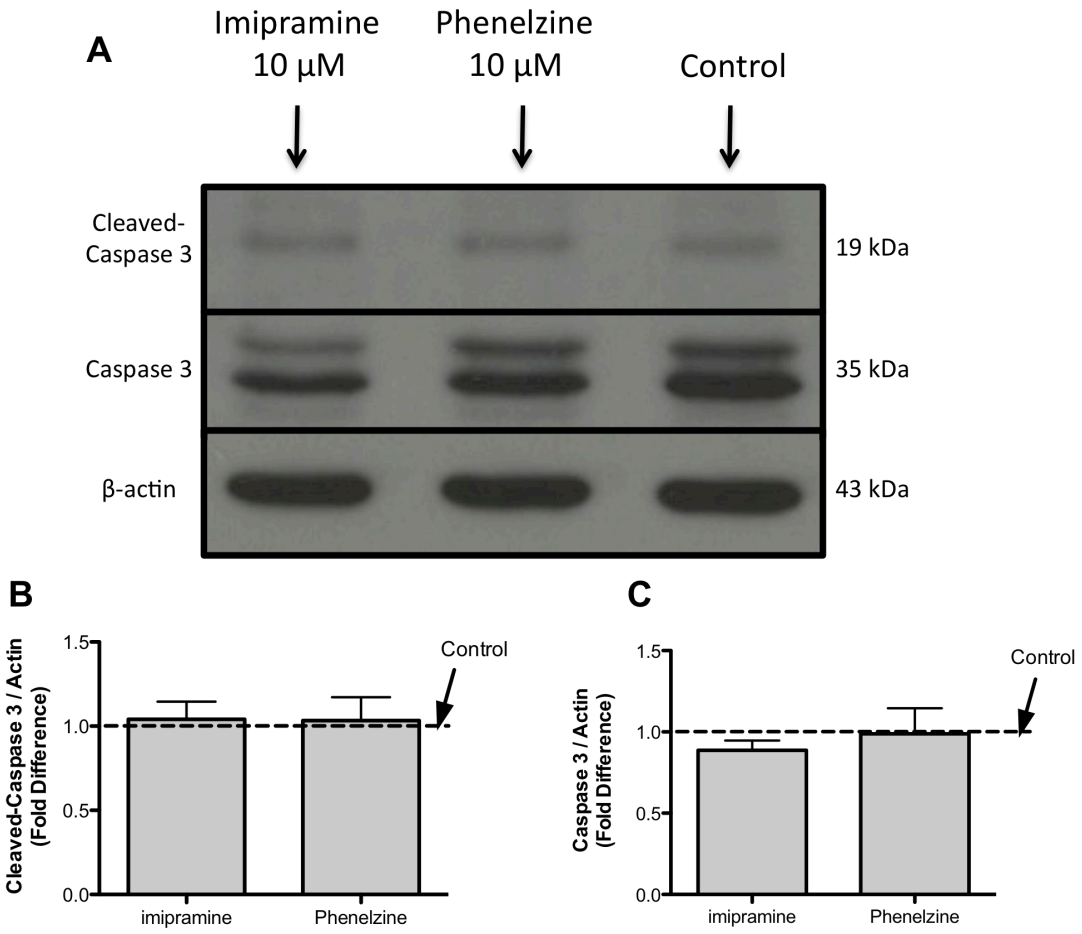
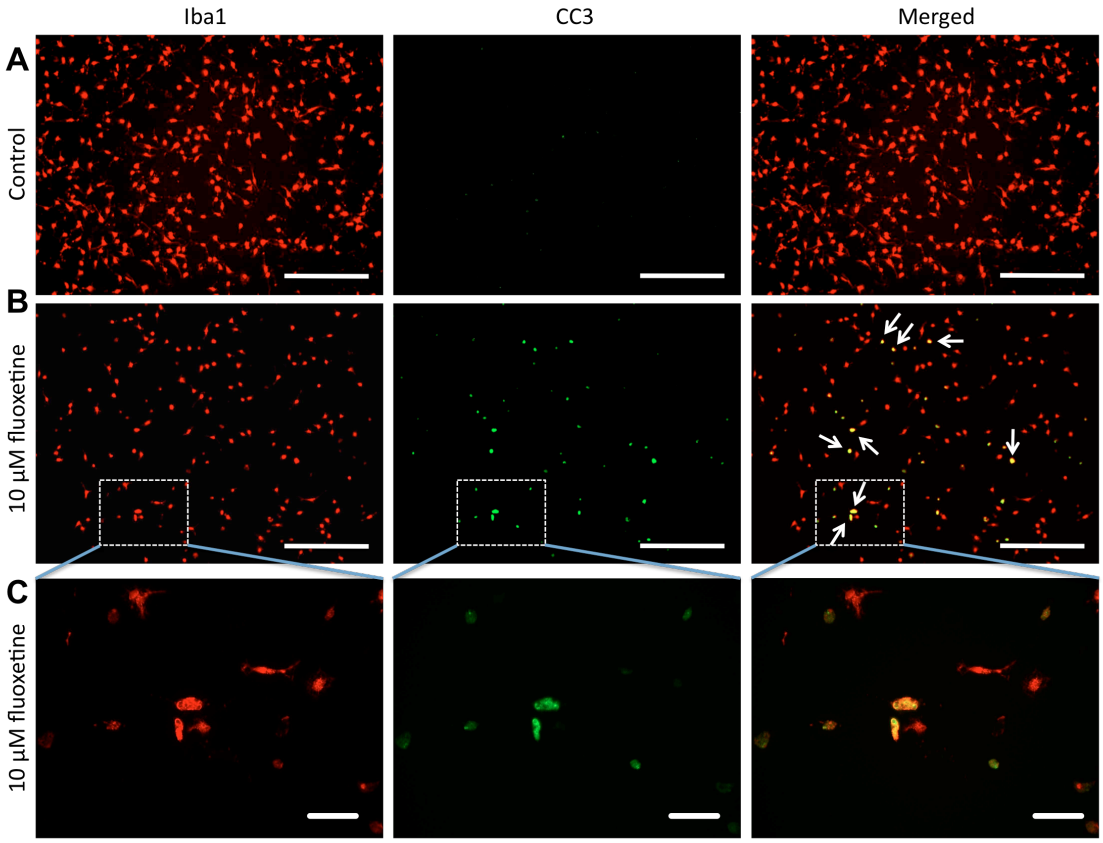
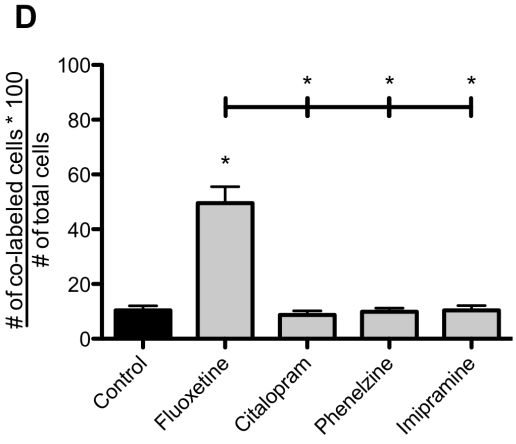


Figure 5-4 – Western Blot analysis for cleaved-caspase 3 and caspase 3 expression in microglial cell lysates following imipramine and phenelzine treatment. A. Imipramine and phenelzine do not alter the expression levels of cleaved-caspase 3 (19 kDa) or caspase 3 (35 kDa) compared to non-treated controls. β-actin (43 kDa) expression was measured as a loading control. **B and C.** Histograms displaying cleaved caspase 3 (**B**) and caspase 3 (**C**) expression from western blot analysis, n = 5. No significance between the treatment groups was found.


Iba1
CC3
Merged
A
Control
B
10 μM fluoxetine
C
10 μM fluoxetine


D
# of co-labeled cells * 100
# of total cells
100
80
60
40
20
0
*
*
*
*
Control
Fluoxetine
Citalopram
Phenelzine
Imipramine

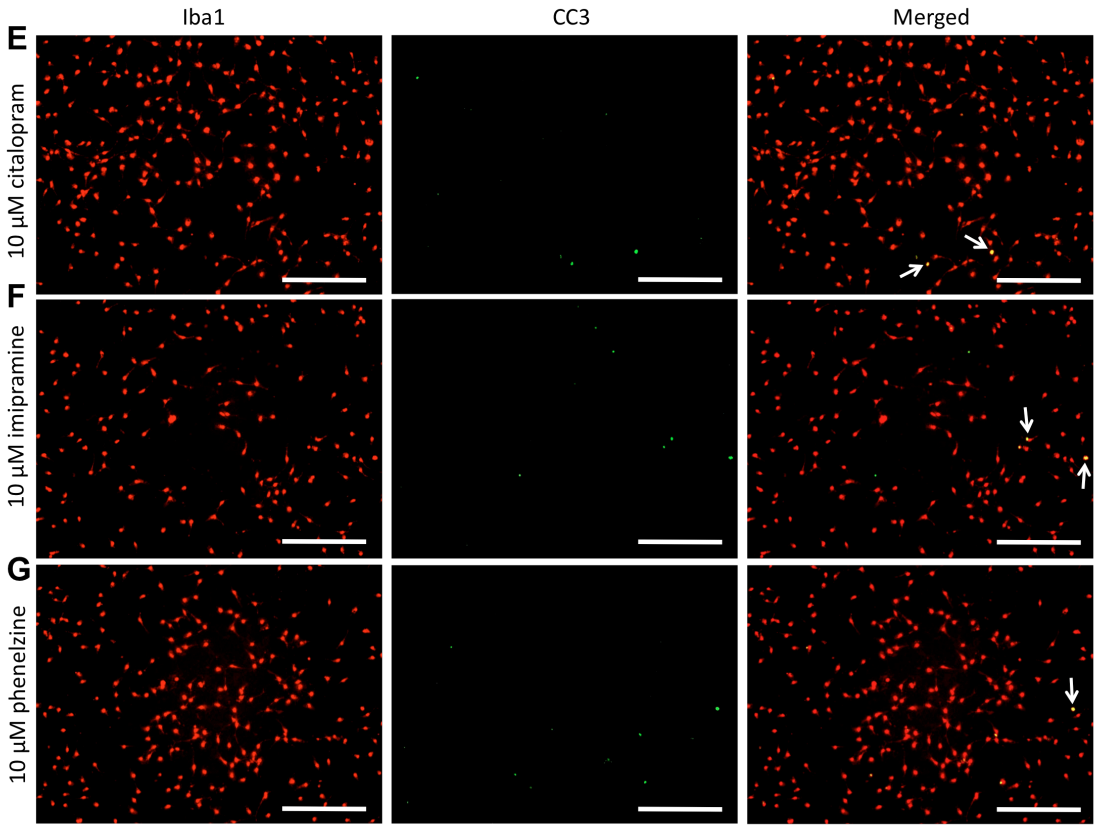
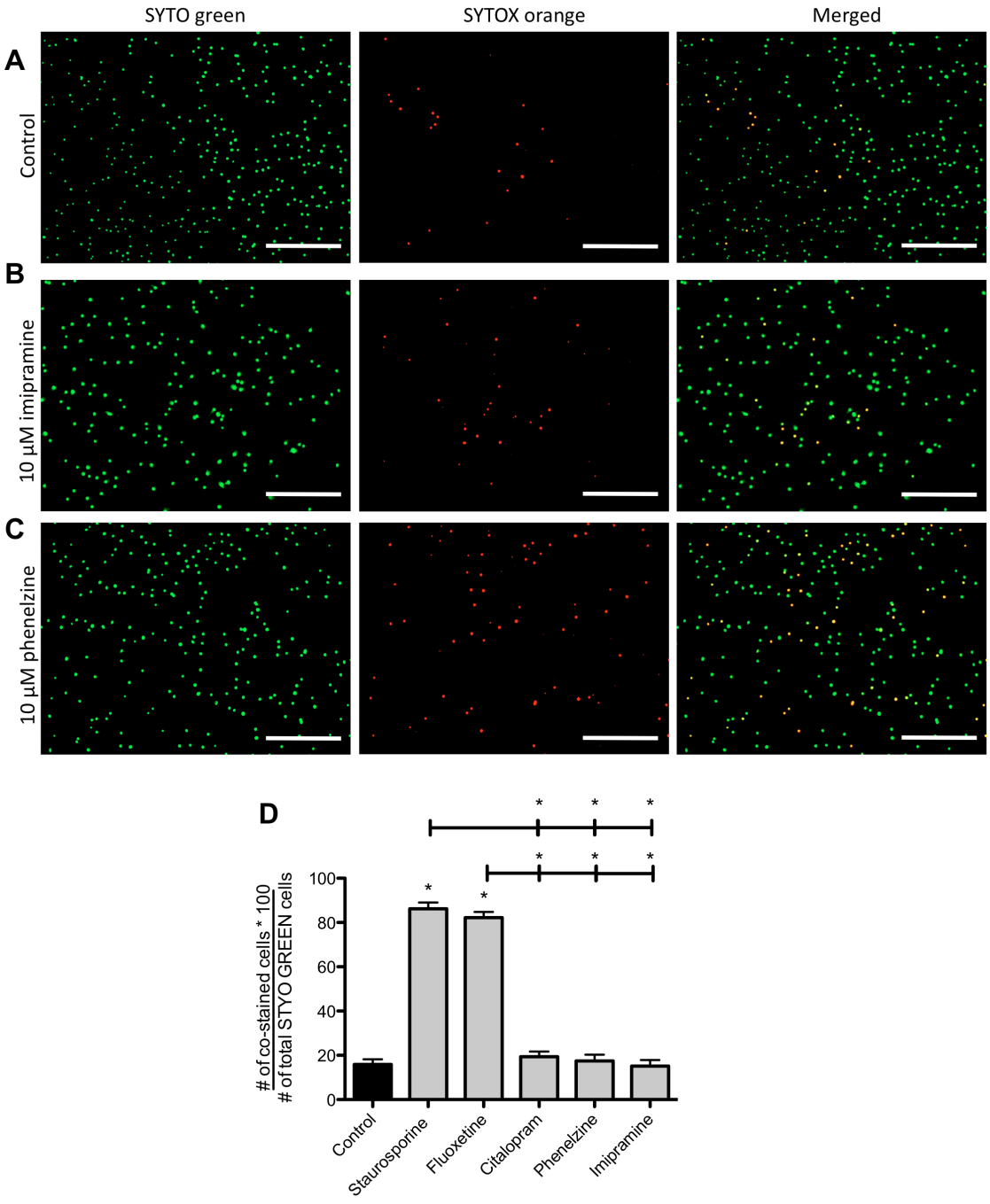


Figure 5-5 – Immunofluorescent photomicrographs showing Iba1 and cleaved-caspase 3 (CC3) expression in antidepressant-treated non-activated microglia. Fluoxetine (**B**) significantly increases the number of cells co-expressing Iba1 (red) and CC3 (green) in microglia compared to non-treated controls (**A**). **C.** Blown up photomicrographs (40 X) from 10 X photomicrographs (**B**). Citalopram (**E**), imipramine (**F**), and phenelzine (**G**) do not increase the number of cells co-expressing Iba1 and CC3 in microglia compared to non-treated controls (**A**). **D.** Histogram displaying the percentage of co-labeled microglia for Iba1 and CC3 across different treatment conditions. Fluoxetine was the only treatment found to significantly increase CC3 expression in microglia compared to control. Scale bar = 80 µM, 10X objective and 50 µM, 40X objective. For each condition n = 4, each n represents pictures taken from 9 fields per well. Statistical significance, * < 0.05 (comparisons to control). Examples of co-expressing microglia are shown in merged images with arrows.


SYTO green
SYTOX orange
Merged
A
Control
B
10 µM imipramine
C
10 µM phenelzine
D
# of co-stained cells * 100
# of total STYO GREEN cells
100
80
60
40
20
0
Control
Staurosporine
Fluoxetine
Citalopram
Phenelzine
Imipramine

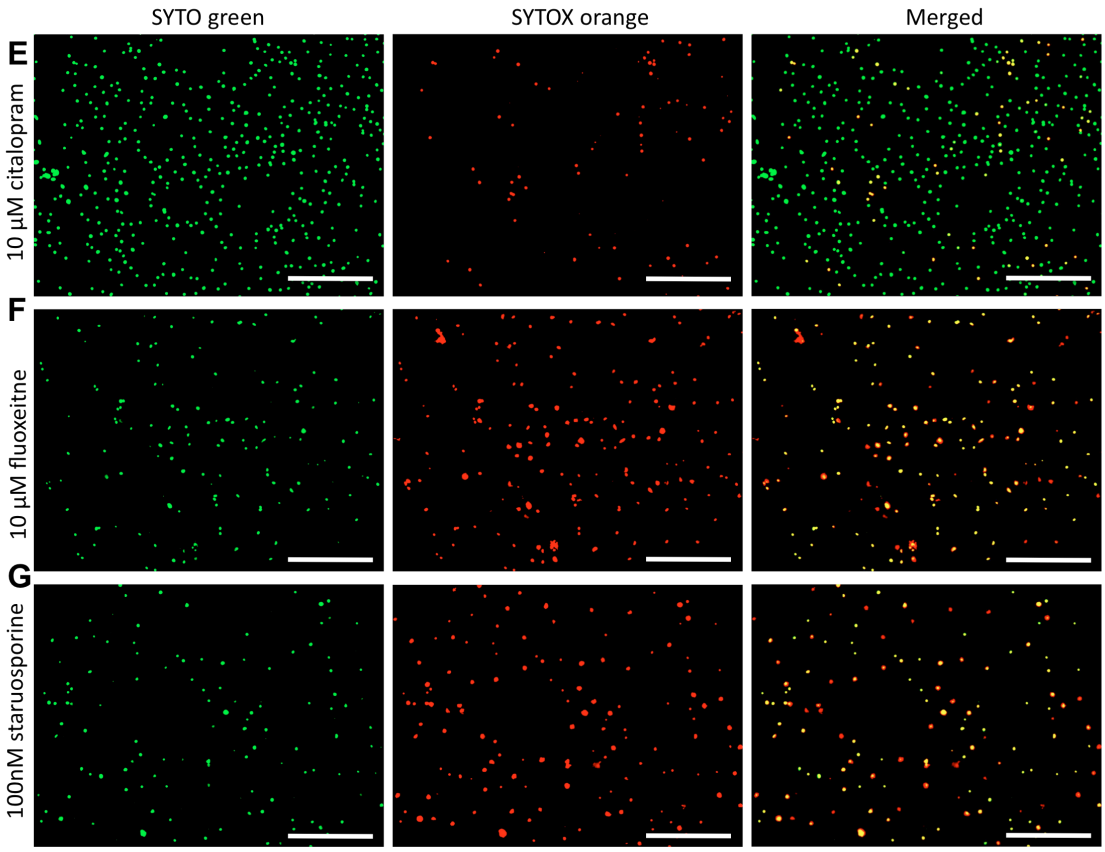


Figure 5-6 – Immunofluorescent photomicrographs showing live/dead staining of antidepressant-treated non-activated microglia. Imipramine (**B**), phenelzine (**C**), and citalopram (**D**) do not increase the number of cells co-stained with the live (SYTO green) and the dead stain (SYTOX orange) in microglia compared to non-treated controls (**A**)**.** Fluoxetine (**F**) significantly increases the number of microglial cells co-stained with the live and dead stain compared to non-treated controls (**A**). **G.** Staurosporine, a postive apoptosis-inducing control, showed similar increases in the number of microglial cells co-stained with the live and dead stain compared to fluoxetine-treated microglia. **D.** Histogram displaying the percentage of co-stainted microglia for the live and dead stains across different treatment conditions. Fluoxetine and staurosporine were the only treatment groups found to significantly increase co-staining in microglia compared to controls. Scale bar = 80 µM, 10X objective. For each condition n = 4; each n represents pictures taken from 9 fields per well. Statistical significance, * < 0.05 (comparisons to control and comparisons between treatment groups).

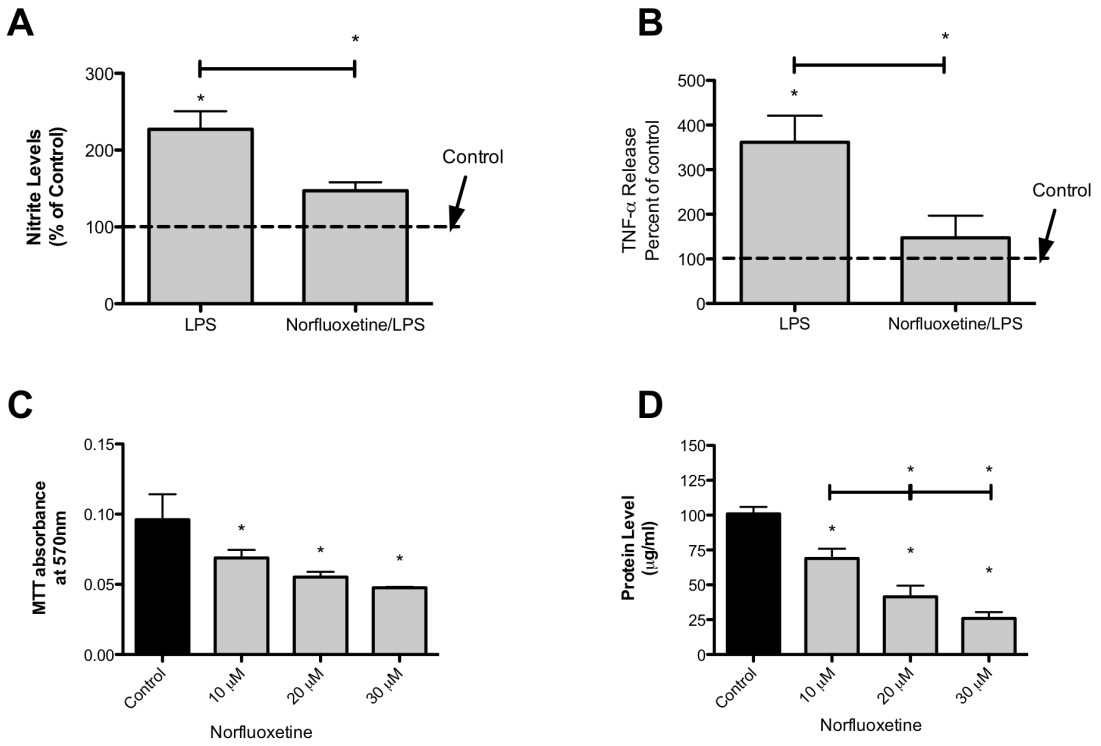


Figure 5-7 – Histograms displaying norfluoxetine effects on the release of pro-inflammatory factors from activated microglia and on cell viability. Norfluoxetine significantly attenuates the release of NO (**A**) and TNF-α (**B**) from LPS-activated microglia, n = 10. Norfluoxetine decreases microglial cell viability (**C**) and protein levels (**D**) within cell lysates at all doses tested, n=10. Statistical significance, * < 0.05 (comparisons to control and comparisons between treatment groups).

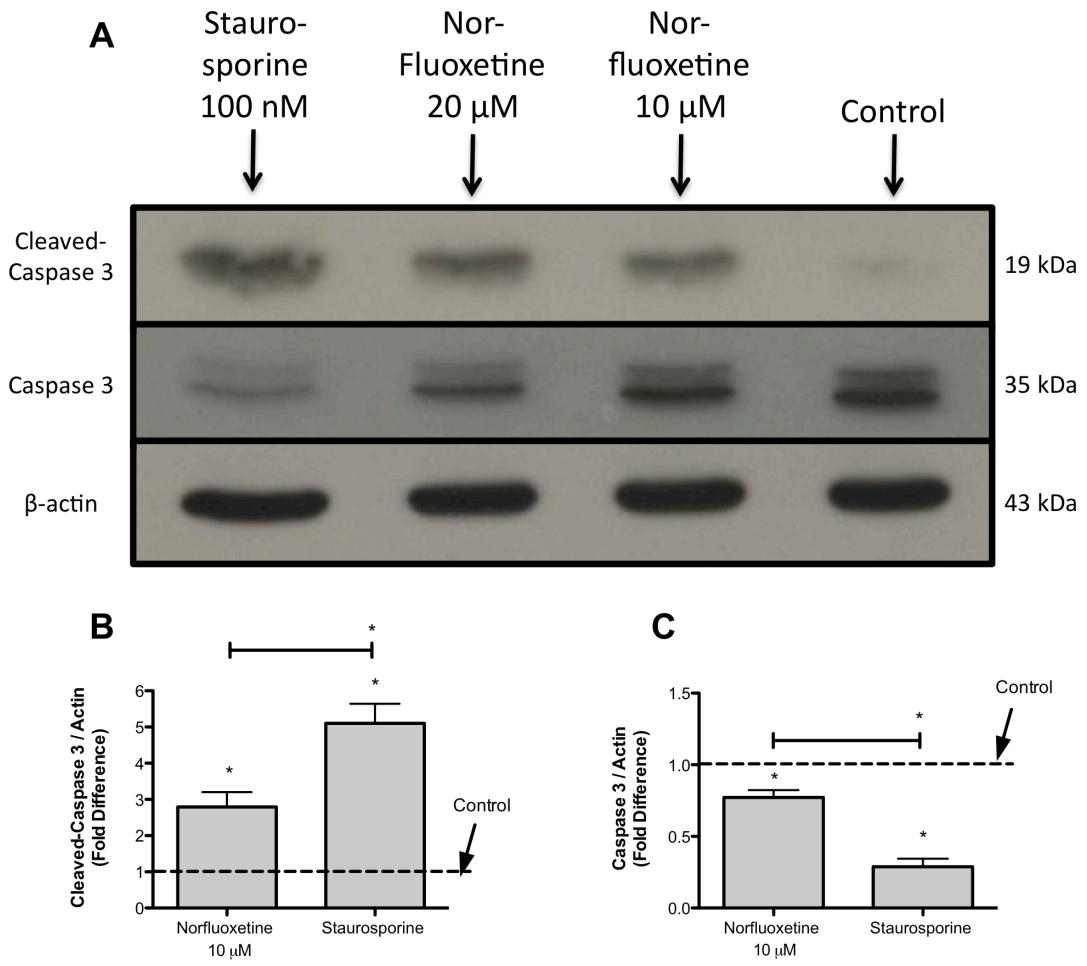
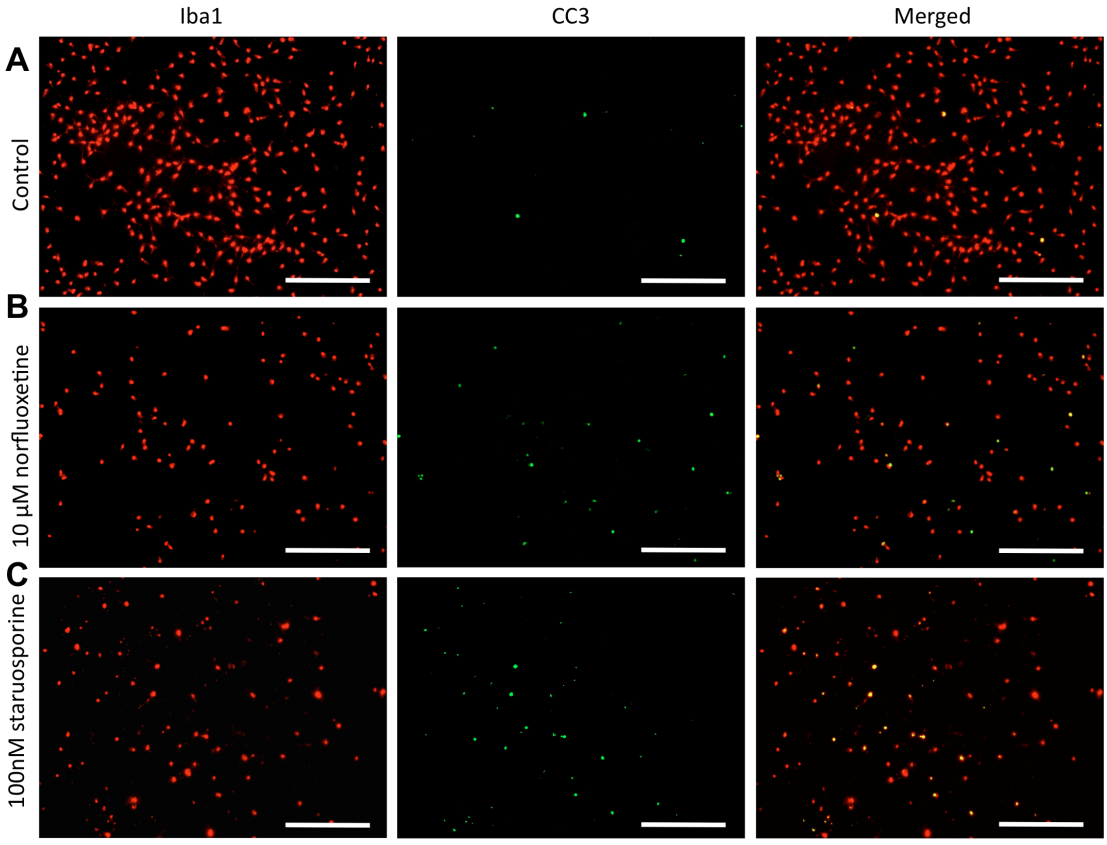


Figure 5-8 – Western Blot analysis for cleaved-caspase 3 and caspase 3 expression in microglial cell lysates following norfluoxetine treatment. A. Staurosporine (a positive control) and norfluoxetine (doses of 10 and 20 µM) increased the expression of cleaved-caspase 3 (19 kDa) and decreased the expression of caspase 3 (35 kDa) compared to non-treated controls. β-actin (43 kDa) expression was measured as a loading control. **B and C.** Histograms displaying cleaved caspase 3 (**B**) and caspase 3 (**C**) expression from western blot analysis. The increase in cleaved-caspase 3 expression and the decrease in caspase 3 expression was greater in staurosporine-treated groups compared to the norfluoxetine-treated groups, n = 5. Statistical significance, * < 0.05 (comparisons to control and comparisons between treatment groups).


Iba1
CC3
Merged
A
Control
B
10 µM norfluoxetine
C
100nM staruosporine

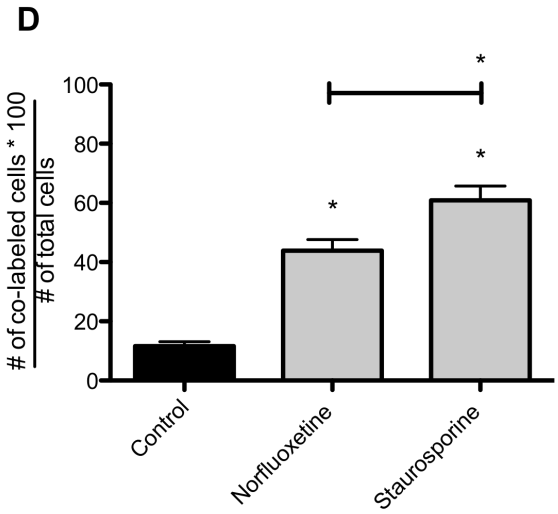
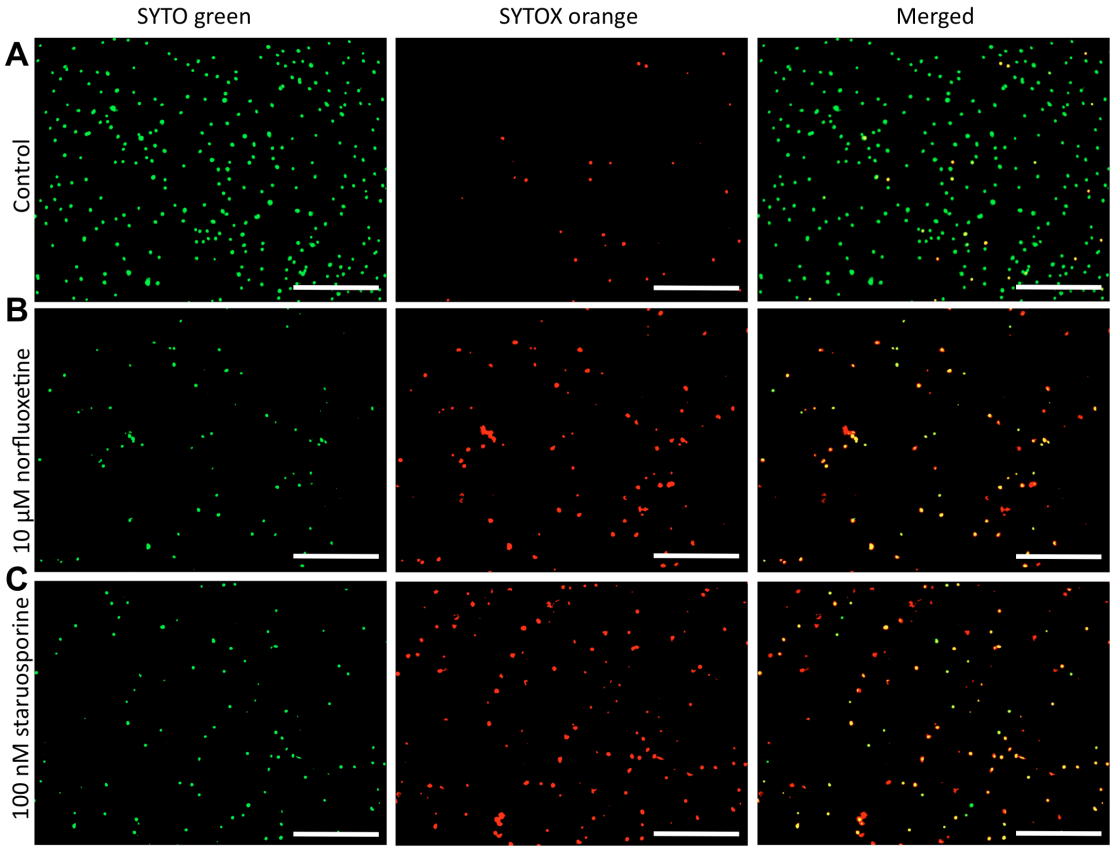

D
# of co-labeled cells * 100
# of total cells
100
80
60
40
20
0
*
*
*
Control
Norfluoxetine
Staurosporine

Figure 5-9 – Immunofluorescent photomicrographs showing Iba1 and cleaved-caspase 3 (CC3) expression in norfluoxetine-treated non-activated microglia. Norfluoxetine (**B**) significantly increased the number of cells co-expressing Iba1 (red) and CC3 (green) in microglia compared to non-treated controls (**A**). **C.** Staurosporine significantly increased the number of cells co-expressing Iba1 (red) and CC3 (green) in microglia compared to non-treated controls. **D.** Histogram displaying the percentage of co-labeled microglia for Iba1 and CC3 across different treatment conditions. Both norfluoxetine and staurosporine significantly increased CC3 expression in microglia compared to control with staurosporine having a greater effect than norfluoxetine. Scale bar = 80 μM, 10X objective. For each condition n = 4, each n represents pictures taken from 9 fields per well. Statistical significance, * < 0.05 (comparisons to control).


SYTO green
SYTOX orange
Merged
A
Control
B
10 µM norfluoxetine
C
100 nM staruosporine

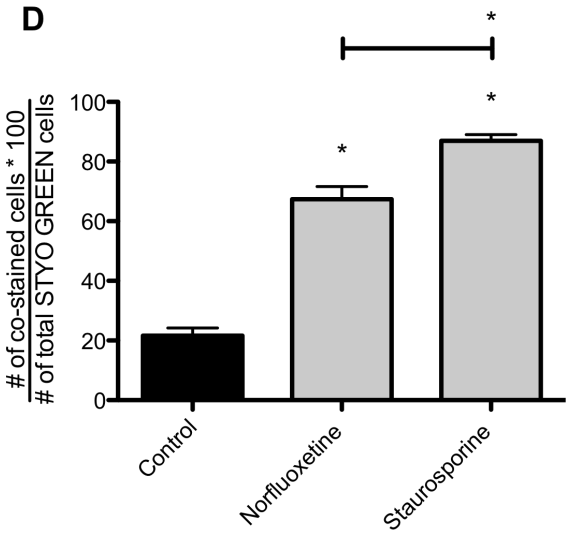

D
# of co-stained cells * 100
# of total STYO GREEN cells
100
80
60
40
20
0
*
*
*
Control
Norfluoxetine
Staurosporine

Figure 5-10 – Immunofluorescent photomicrographs showing live/dead staining of norfluoxetine-treated non-activated microglia. Norfluoxetine (**B**) increased the number of cells co-stained with the live (SYTO green) and the dead stain (SYTOX orange) in microglia compared to non-treated controls (**A**)**.** Staurosporine (**C**) significantly increased the number of microglial cells co-stained with the live and dead stain compared to non-treated controls (**A**). **D.** Histogram displaying the percentage of co-stained microglia for the live and dead stains across different treatment conditions. Both norfluoxetine and staurosporine significantly increased live/dead co-staining in microglia compared to control with staurosporine having a greater effect than norfluoxetine. Scale bar = 80 μM, 10X objective. For each condition n = 4, each n represents pictures taken from 9 fields per well. Statistical significance, * < 0.05 (comparisons to control and comparisons between treatment groups).

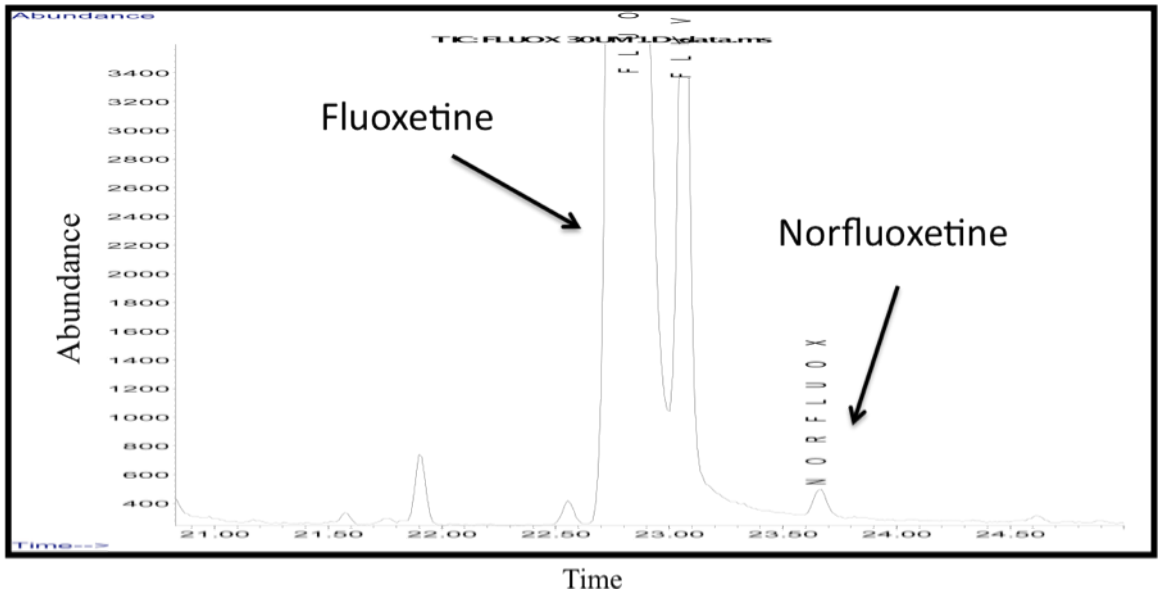


Figure 5-11 – A gas chromatograph (GC) trace of the levels of fluoxetine and norfluoxetine in the medium of fluoxetine-treated non-activated microglia. GC revealed displayed high levels of fluoxetine in the medium of fluoxetine-treated microglia, whereas norfluoxetine levels were virtually nonexistent. Therefore, microglia were not converting fluoxetine to norfluoxetine in culture, n = 3.

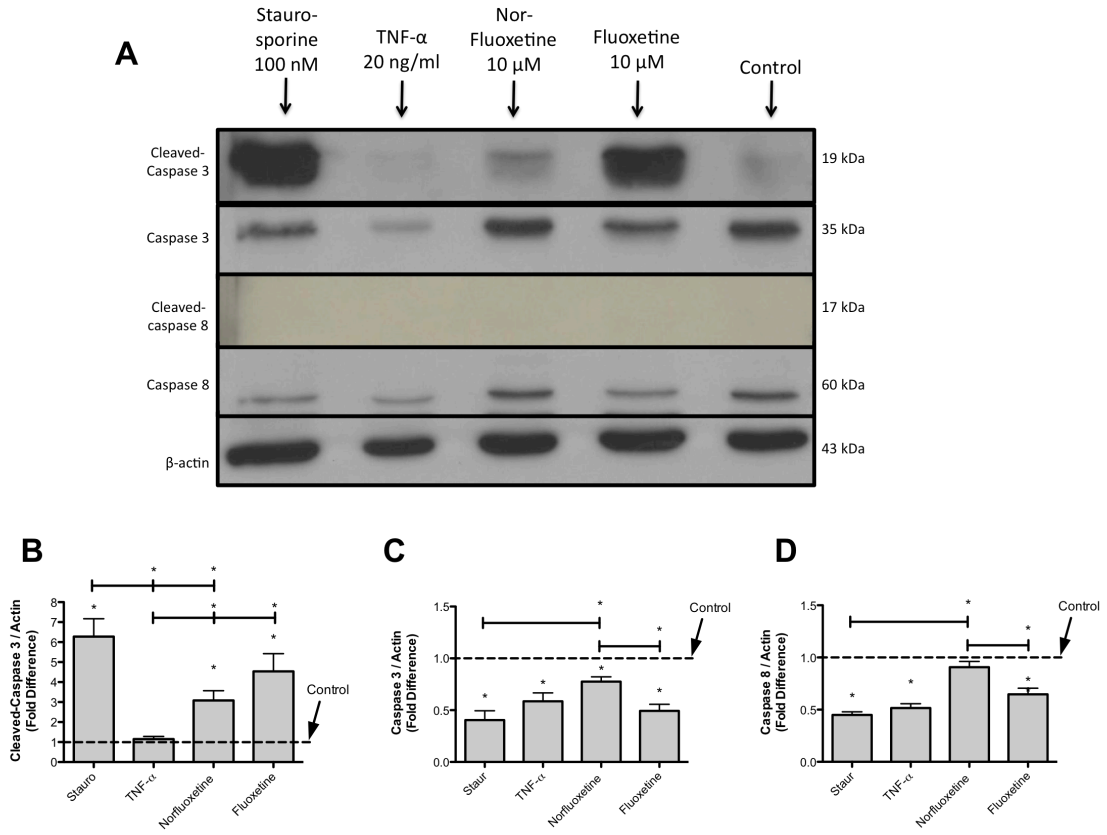


Figure 5-12 – Western Blot analysis for cleaved-caspase 3, caspase 3, cleaved-caspase 8, and caspase 8 expression in microglial cell lysates following antidepressant treatments. A. Staurosporine (a positive control), norfluoxetine, and fluoxetine increased the expression of cleaved-caspase 3 (19 kDa) and decreased the expression of caspase 3 (35 kDa) compared to non-treated controls. TNF-α treatment had no effect on cleaved-caspase 3 expression but did decrease caspase 3 expression compared to non-treated controls. Caspase 8 (60 kDa) expression was decreased in staurosporine, TNF-α, and fluoxetine-treated groups, but norfluoxetine did not have this effect. No treatment group showed the expression of cleaved-caspase 8 (19 kDa). β-actin (43 kDa) expression was measured as a loading control. **B, C, and D.** Histograms displaying cleaved caspase 3 (**B**), caspase 3 (**C**), and caspase 8 (**D**) expression from western blot analysis, n = 5. Statistical significance, * < 0.05 (comparisons to control and comparisons between treatment groups).

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CHAPTER 6

General Discussion

6.1. Summary of questions

This thesis investigated the interactions between the effect of antidepressants on inflammatory signaling within the brain and on cortical neuronal viability following an ischemic insult. We were interested in the following: 1) Can antidepressants modulate the inflammatory profile of LPS-activated microglia and how does this modulation compare across several classes of antidepressants? 2) Can antidepressants attenuate the loss in the viability of ischemic-injured cortical neurons co-cultured with LPS-activated microglia and if so, is the mechanism through attenuating the inflammatory release profile of microglia? 3) What are the main factors released from LPS-activated microglia that cause a decrease in the viability of injured cortical neurons? 4) Finally, what is the mechanism behind the attenuation in the release of these main factors from activated microglia following antidepressant treatment?

6.2. Can antidepressants modulate the inflammatory profile of LPS-activated microglia and how does this modulation compare across several classes of antidepressants?

Previous studies have investigated the effects of antidepressants on the release of inflammatory factors from activated microglia (Ha et al. 2006; Obuchowicz et al. 2006; Hashioka et al. 2007). However, to my knowledge, there are no studies that compare the effects between several classes of antidepressants on the release of these factors. Therefore, we decided to investigate the effects of six antidepressant drugs, from three separate classes, on the inflammatory profile of microglia: the TCAs (imipramine and

clomipramine), the MAOIs (phenelzine and TCP), and the SSRIs (fluoxetine and citalopram).

6.2.1. Purity of isolated microglia cultures

We were confident in the purity of the primary microglial cultures used in the experiments for this thesis. These cultures displayed no labeling for astrocytes, oligodendrocytes, or neurons and clearly showed the presence of microglia. To rule out other contaminating cell types such as endothelial cells, fibroblasts, or ependymal cells, we co-labeled all the cells with a microglial marker (Iba1) and a nuclear marker (DAPI) and found that over 99% of the cells co-labeled for both these markers. Contaminating cell types would label with DAPI and not Iba1, and the presence of these cells was minimal.

6.2.2. Antidepressant effects on the release of pro-inflammatory factors from non-activated and LPS-activated microglia

We found that all six antidepressant drugs tested did not effect the release of NO, TNF-α, or IL-1β from non-activated microglia. When the cultured microglia were activated with LPS, we found a significant release of these pro-inflammatory factors into the medium. The release of NO and IL-1β was attenuated by all six antidepressant drugs at treatments of 10 μM, whereas all but phenelzine and clomipramine attenuated the release of TNF-α at this concentration. An argument could be made that these antidepressants are not equipotent and it may be possible that phenelzine and clomipramine would attenuate TNF-α release from activated microglia if these concentrations were increased; however,

based on dose-response studies, phenelzine and clomipramine treatments as high as 40 μM did not attenuate this TNF-α release. These results show that the unequal potency of these antidepressant drugs at 10 μM does not explain the lack of attenuation in TNF-α release from activated microglia following phenelzine or clomipramine treatments.

There are various studies in the literature that support and also contradict our results regarding the anti-inflammatory properties of imipramine, clomipramine, and citalopram on activated microglia. Studies have shown that imipramine and clomipramine attenuate the release of NO and TNF-α from activated microglia (Hashioka et al. 2007; Hwang et al. 2008; Song et al. 2012), which support the results in our study. Citalopram (an SSRI) at a concentration of 10 μM has been shown to attenuate the release of TNF-α from activated microglia (Tynan et al. 2012), similar to what is seen in our study, but Tynan et al. (2012) did not find any significant decreases in NO release at any concentrations of citalopram tested, which ranged from 0.1 to 35 μM . Our study showed that citalopram decreased the release in NO at concentrations similar to those used by Tynan et al. (2012). To my knowledge, these are the only two studies that have measured the effect of citalopram on the release of NO from activated microglia; therefore, further studies are required to verify or disprove this drug's capabilities in attenuating microglial NO release.

The fluoxetine data in our study showed some results contradictory to another study in the literature. Ha et al. (2006) reported that fluoxetine increased IL-1β and TNF-α mRNA levels, NO release, and expression of iNOS in cultured non-activated microglia,

whereas we found no significant increases in the release of NO, TNF-α, or IL-1β from non-activated microglia. One explanation for these differences is that their study failed to measure the protein levels of IL-1β and TNF-α in the medium, whereas our study failed to measure the mRNA levels of these factors in cell lysates. It is possible that these factors are transcribed within microglia into mRNA but not yet translated into proteins or released into the medium following 24 hrs post-treatment with fluoxetine. When we measured NO release from non-activated microglia following fluoxetine treatment, we found no significant increase, whereas Ha et al. (2006) found increases in NO production and release. This finding by Ha et al. (2006) seems peculiar because historically, NO release from microglia has been conceptualized as a pro-inflammatory factor involved in various disorders and diseases. The ability of fluoxetine to increase NO release from physiologically resting microglia may sound counter-intuitive since the drug is used to treat depression. However, a study by Cardenas et al. (2005) indicated that NO may have a dual role in the brain specifically regarding neurogenesis. The effects of NO on neurogenesis may depend on the type of NOS used for its synthesis. *In vivo* studies have shown that NO produced from inducible and endothelial isoforms of NOS stimulate neurogenesis (Reif et al. 2004) whereas NO produced from the neuronal isoform reduces neurogenesis (Moreno-Lopez et al. 2004). The predominant form of NOS in microglia is the inducible isoform. Therefore, fluoxetine-induced NO release by microglia may be beneficial for neurogenesis. However, it is possible that the over-expression of NO from activated microglia stimulates the production of NO from neurons. Neuronal-derived NO may possibly overshadow the beneficial effects of resting microglial-derived NO on neurogenesis. It is important to keep in mind that fluoxetine treatment does not elevate

the release of NO to the extent seen with the microglial activators LPS or IFN-γ. Therefore, the slight but significant increase in NO release from microglia after fluoxetine treatment in the study conducted by Ha et al. (2006) may be beneficial within the brain. Furthermore, the fluoxetine study by Tynan et al. (2012) and our study investigated the drug's effects on activated microglial cultures, and both studies show that fluoxetine attenuates NO release from the activated state. These observations support the importance of NO levels in the CNS. High levels of NO released by activated microglia may result in toxicity by stimulating NO synthesis in neurons and low to moderate levels of NO released from resting microglia may promote neurogenesis. However, further studies are necessary to investigate the role of NO in depression and following antidepressant treatment.

To my knowledge, there has been only one other study that has investigated the effects of phenelzine on the release of pro-inflammatory factors from activated microglia and this study contradicts the findings in this thesis. Chung et al. (2012) reported that a high concentration of phenelzine (50 μM) increases the production of NO, TNF-α and IL-6 from LPS-activated microglia compared to non-activated cells, whereas our data showed that phenelzine concentrations of 40 μM or less decreased this production of NO and had no effect on TNF-α release from these activated cells. A number of factors may explain the discrepancies between these two studies. First, our study never investigated the concentration of phenelzine at 50 μM and it is possible that if we had, we may have observed the increase in the release of these pro-inflammatory factors from activated microglia. However, Chung et al. (2012) did test other concentrations of phenelzine,

including 1 and 10 μM, where they found no significant increase or decrease in the release in NO from activated microglia, whereas our study found that phenelzine concentrations of 5, 10, 20, 30, and 40 μM decreased this release in NO. However, both studies did show that 10 μM phenelzine caused no significant changes in the release of TNF-α. The differing results in NO data between these two studies can be due to a number of factors. First, it appears the group presented their nitrite release (an indirect measure of NO) without accounting for the amount of cells or levels of protein within each condition. The increase in NO release from activated microglia after phenelzine treatment may just be a result of a greater number of cells in these conditions. In our study, we normalized the nitrite data by dividing these levels by total protein levels in each condition, accounting for this possibility. Second, this group used Western blotting to study the increase in iNOS expression in activated microglia after the treatment with 50 μM of phenelzine; however, actin loading, a control for equal loading of protein across conditions, seems to be inconsistent across different concentrations of phenelzine, with 50 μM appearing to have the greatest levels of total protein. Therefore, the blot that showed an increase in iNOS expression after 50 μM phenelzine treatment may be an artifact of unequal loading in protein levels. Furthermore, this group failed to produce a histogram reporting data across various experiments with the expression of iNOS normalized over actin as a control. Finally, LPS concentrations in these two studies can be another source of variability. Chung et al. (2012) activated the microglia in their study with 0.2 μg/ml LPS, whereas in our study, an LPS concentration of 1 μg/ml was used. Their study measured approximately a 2-fold increase in NO release following LPS activation, whereas our study found a 3-fold increase. Phenelzine's effects on NO release

from LPS-activated microglia may be dependent on the activation state of the cells. In our study we measured the release of NO following the activation of microglia with 1 μg/ml. We found that although the differences between 0.2 and 1 μg/ml LPS concentrations on this NO release were not significant, there was an increase with 1 μg/ml, whereas their study did not investigate this difference.

To my knowledge, we are the first to investigate the effects of tranylcypromine (a MAOI) on the release of inflammatory factors from activated and non-activated microglia. We found that tranylcypromine attenuated the release of NO, TNF-α, and IL-1β from activated microglia, but further studies are required to verify or disprove this drug's capabilities in attenuating the release of these pro-inflammatory factors from activated microglia.

Our data suggest that the attenuation in NO, TNF-α, or IL-1β release from activated microglia after antidepressant treatment is not dependent on the class of the drug. All the antidepressant drugs tested in this study attenuated NO and IL-1β release, showing no class-dependent actions of the drugs; however, phenelzine (MAOI) and clomipramine (TCA), from two separate classes, did not attenuate the release of TNF-α. When we look at the antidepressants TCP (a MAOI, same class of drug as phenelzine) and imipramine (a TCA, same class of drug as clomipramine), these drugs do attenuate the release of TNF-α from activated microglia. Therefore, the class of antidepressant does not determine the attenuation in NO, TNF-α, or IL-1β release from LPS-activated microglia.

6.3. Can antidepressants attenuate the loss in the viability of OGD-injured cortical neurons and, if so, is this mediated through the attenuation of the inflammatory release profile of microglia?

Depression and stroke incidence are highly correlated with one another. Patients diagnosed with depression have a greater likelihood of having a stroke later in life, whereas the most prevalent disorder post-stroke is depression (Smoller 2011; Esparrago Llorca et al. 2012; Li et al. 2012; Loubinoux et al. 2012). The most commonly prescribed treatment for post-stroke depression is antidepressant drugs and they have been shown to alleviate the depressive symptoms and sleep disturbances in these patients (Gothe et al. 2012; Sunami et al. 2012). Since antidepressant drugs are often prescribed after a stroke, we investigated if these drugs could affect the viability of ischemic-injured cortical neurons *in vitro*. We decided to investigate these effects specifically on cortical neurons, because the cortex is highly susceptible to damage following MCAo, a common occlusion causing a stroke (Kronenberg et al. 2012). In addition, antidepressant effects on the viability of injured neurons from other regions of the brain such as the hippocampus and substantia nigra have been studied (Jin et al. 2009; Chung et al. 2011; Zhang et al. 2012), but to my knowledge, no study has investigated these effects on neurons from the cortex.

6.3.1. Co-culture model

As a model of ischemic injury *in vitro*, primary cortical neurons underwent OGD for various times. OGD is a relevant model of ischemic injury in the brain because it mimics the depletion of oxygen and glucose, both essential for aerobic metabolism, after the loss

in blood flow (Guo et al. 2013; Ju et al. 2013). In this thesis, cortical injury was measured with the MTT assay, a measure of cell viability. MTT is a dye that is converted to an insoluble purple product by functional mitochondria in cells (Dai et al. 2012). We found that 40 min of OGD was an effective time point to cause a reduction in the viability of neurons by 40%. After OGD-injury, neurons were placed in co-cultures with LPS-activated or non-activated microglia, and we found a further decrease in the viability of these injured neurons when placed in co-cultures with LPS-activated microglia, whereas this effect was not observed with non-activated microglia. The neurons and microglia were situated within the co-culture in such a way that these cells did not contact each other, but factors released from these cells could affect the other. Microglia were pre-treated with LPS for 24 hrs and prior to being placed in these co-cultures these cells were washed thoroughly with medium to remove residual LPS. Some may question whether these cultures may still contain residual LPS even after the washes and if this LPS may be the factor that further reduces the neuronal viability in the co-cultures. Primary mouse neuronal cultures have been shown to express TLR4 receptors and LPS induces a strong release of the chemokine ligands 1 and 5, TNF-α, and IL-6 from these cells (Leow-Dyke et al. 2012); therefore, it is possible that the primary cortical neurons cultured in this study also express the TLR4 receptor. We tried to account for this by performing control experiments following the washing of the LPS-treated microglia. We washed the wells in which LPS-activated microglia were cultured four times with fresh medium, and after each wash, we placed this medium onto non-activated microglia and measured the release of NO and TNF-α from these cells. The media from the first wash yielded an increase in the release of NO and TNF-α from non-activated

microglia, showing that this wash medium contained LPS, which activated this new population of cells. The media from the second, third, or fourth washes yielded no increases in the release of NO and TNF-α from non-activated microglia, showing that these wash media contained insufficient LPS to activate a new population of cells. Thus there was minimal contamination of LPS left in the original microglial cultures. We washed these cultures 4 times with fresh medium to ensure that there was no LPS residue in the co-cultures.

LPS is an endotoxin derived from the outer membrane of gram-negative bacteria and activates microglia through the TLR4 receptor (Akira et al. 2001; Lu et al. 2008); it was used to activate the microglia in our study. It is debatable as to how clinically relevant it is to use LPS as a microglial activator *in vitro* since it is not endogenously produced in rodents or humans and the only way LPS can activate microglia is through a bacterial infection or exposure to LPS, whereas following a stroke, many possible endogenous activators may be released from injured cortical neurons that can activate microglia; these activators include ATP, glutamate, and cytokines (Freidin et al. 1992; Renauld and Spengler 2002; Li et al. 2011; Kato et al. 2012). In this study, we chose LPS as an activator of microglia for three primary reasons. First, the TLR4 has been shown to mediate microglial activation and production of inflammatory mediators following hypoxia (Yao et al. 2013). This group found that TLR4 immunofluorescence, TLR4 protein expression, and the expression of pro-inflammatory factors (NO and TNF-α) were enhanced following hypoxia in rat neonatal microglia both *in vivo* and primary cell cultures. siRNA knockdown of TLR4 reduced this hypoxia-induced increase of these

pro-inflammatory factors in cultured cells and inhibition of TLR4 *in vivo* decreased the immunoexpression of these factors. Second, LPS binds to TLR4 and is an effective activator of microglia that induces the release of a wide range of inflammatory mediators often seen in stroke (Vartanian et al. 2011; Yousuf et al. 2012). In the presence of all these factors released from LPS-activated microglia, we were able to utilize compounds to antagonize or neutralize the effects of one factor at a time and determine the importance of that factor on the viability of injured cortical neurons in the co-cultures. Finally, LPS is the most common activator of primary cultured microglia in the literature (Chen et al. 2012; Shimizu et al. 2012) and LPS activation is robust across investigations from various laboratories. We can compare the results from our studies to the findings in the literature to ensure that microglia cultured in this study have similar reactive phenotypes to other studies following LPS activation. It is still imperative that future experiments examine the effects of microglia that have been stimulated with endogenous activators, such as ATP and cytokines, on the viability of ischemia-injured cortical neurons. These endogenous activators released from neurons can stimulate microglia through a wide range of receptors including purinergic, TNF, IL-1, and death-associated receptors (Rappold et al. 2006; Hosmane et al. 2012; Ishikawa et al. 2013), independent of TLR4 signaling, thus activating diverse intracellular cascades. These diverse cascades then lead to the differences in the production and release of inflammatory factors from microglia.

We transferred OGD-injured cortical neurons into co-cultures with non-activated microglia and found no additional decrease in neuronal cell viability after an OGD insult,

whereas neuronal viability was further decreased when these injured neurons were co-cultured with LPS-activated microglia. Based on this result, we conclude that 24 hrs after injury, these neurons do not release sufficient factors to activate microglia to the extent of LPS activation and cause the release of pro-inflammatory factors from microglia, which then can further decrease neuronal viability. Some may argue that this model of neuronal injury and the co-culturing with non- and LPS-activated microglia does not properly represent the *in vivo* conditions of ischemic insult. *In vivo*, ischemia causes the release of factors from neurons that can activate microglia; however, these factors were washed away prior to co-culture and this may explain why the injured neurons do not activate non-stimulated microglia in the co-culture experiments. For instance, injured neurons can release glutamate, which activates the glutamate metabotropic receptor mGlu2, triggering microglial TNF-α release and subsequently greater neuronal death (Taylor et al. 2005). In our study, we focused more on the factors released from LPS-activated microglia that mediated the losses in neuronal viability after an ischemic insult.

Prior to the co-culture experiments, microglia were stimulated with LPS for 24 hrs followed by washes to remove residual LPS. It is speculative that after these washes, microglia no longer remain in the activated state and reduce their release of inflammatory factors compared to the initial 24 hrs of activation. If this were the case, then the once activated microglia would have little to no effect on these injured cortical neurons. However, we observed and measured a further decrease in cortical neuronal viability when these neurons were co-cultured with pre-activated microglia whereas non-activated microglia had no effect on this viability. In addition, we measured NO and TNF-α

release from pre-activated microglia 24 hrs following LPS removal and found that the levels of these factors were not significantly different compared to the levels measured 24 hrs following LPS activation. These results show that pre-activated microglia continue to release factors after LPS is washed from the cultures.

The next step was to investigate if the antidepressant drugs could attenuate the loss in cortical neuronal viability after an OGD insult *in vitro*. Prior to OGD-insult, cultured neurons were pre-treated with 10 μM antidepressants for 30 min. Following OGD, we found that the antidepressants did not attenuate the loss in viability of the injured neurons. Therefore, the antidepressant drugs in this study did not have direct effects on cortical neuron viability after OGD-injury. There are numerous *in vivo* studies reporting that imipramine and fluoxetine induce a decrease in neurodegeneration and an increase in neural stem cell proliferation following ischemic insult (Wang et al. 2008; Li et al. 2009; Schiavon et al. 2010); however, these studies did not investigate if these effects were the direct action of antidepressants on neurons and did not study the possible involvement of other cell types. In our study, we investigated whether the loss in neuronal viability is attenuated through the actions of antidepressants on microglia in co-culture. We found attenuation in the losses in viability of injured cortical neurons when LPS-activated microglia were pre-treated with the antidepressants fluoxetine and citalopram (SSRIs), whereas the other antidepressants tested (imipramine, clomipramine, phenelzine, and tranylcypromine) did not attenuate this loss. An interesting result is that we found no effects of imipramine in attenuating the losses in neuronal viability after co-culture or OGD-insult, whereas other studies have (Hwang et al. 2008; Schiavon et al. 2010; Van

Bokhoven et al. 2011). The region where neurons were assessed or cultured from can explain the differences in the results between these studies and our study. For instance, Hwang et al. (2008) and Schiavon et al. (2010) investigated the viability of ischemic-injured neurons following imipramine treatment in the hippocampus and Van Bokhoven et al. (2011) studied imipramine's effects on neuroblastoma cells in co-cultures with microglia *in vitro*. In our study, we investigated the effects of antidepressants on the viability of OGD-injured cortical neurons. There may be a major difference between the ischemic vulnerability of different regions of the brain. As an example, the CA1 region of the hippocampus, the limbic system, cerebellar Purkinje cells, and medium-sized neurons of the striatum may suffer irreversible injury after global ischemia of as short as 5 min (Kirino 1982) whereas other nerve cell populations such as certain layers of the cortex, survive this ischemic insult of as long as 1 h (Hossmann 1988). Another explanation can be that these contradictory studies investigated imipramine's effects on neuronal viability after ischemic insult *in vivo* where many more cell types are involved in determining the fate of neurons, compared to the *in vitro* conditions in our study, which only consist of neurons and microglia.

6.4. What are the main factors released from LPS-activated microglia that cause a decrease in the viability of OGD-injured cortical neurons?

The next step was to investigate what mediators released from activated microglia are main factors in determining the loss in neuronal viability of OGD-injured cortical neurons in co-culture. We hypothesized that there must be certain factors that are released from LPS-activated microglia that mediate this loss in neuronal viability based

on the results in our previous experiments. We base our hypothesis on two major findings. First, of the six antidepressants tested, we found that the treatments of fluoxetine and citalopram (10 μM) attenuated the loss in the viability of injured-cortical neurons by decreasing the inflammatory profile of activated microglia in co-culture, whereas the remaining four antidepressants (imipramine, clomipramine, phenelzine, and tranylcypromine) did not have this effect. Second, all the antidepressants (10 μM) tested attenuated the release of the pro-inflammatory mediators NO, TNF-α, and IL-1β from LPS-activated microglia, with the exception of the insignificant effects on TNF-α release by phenelzine and clomipramine treatments. We concluded that microglial-released NO, TNF-α, and IL-1β were not the main factors causing the losses in viability OGD-injured cortical neurons in co-culture because the antidepressants imipramine and tranylcypromine, which had been shown to attenuate the release of these factors, had no effect on attenuating this loss in viability. We set out to find the main factors by measuring other mediators that can be released from microglia, namely the amino acids glutamate and D-serine, BDNF, GDNF, and IL-4 (Miwa et al. 1997; Chen et al. 2012; McMullan et al. 2012).

6.4.1. GDNF, IL-4, and BDNF release from microglia

We found that the activation with LPS did not significantly alter the release of GDNF or IL-4 from cultured microglia. Studies have shown that GDNF is decreased following activation of glial cultures (Matsushita et al. 2008) and is increased in these cultures following anti-inflammatory treatments (Ossola et al. 2011; Iravani et al. 2012), but these cultures contain astrocytes in addition to microglia. Thus, the GDNF measured in these

glial culture studies may reflect the effect of LPS in suppressing GDNF release from astrocytes rather than microglia. In our study, the cultures consist of pure microglia, and without the presence of astrocytes, GDNF release may not be induced by LPS activation. In another study involving pure microglial cultures, Mizuno et al. (2004) found that LPS did not increase the expression or release of GDNF; however, following treatments with the anti-inflammatory compound ibudilast (a cyclic AMP phosphodiesterase inhibitor), GDNF mRNA expression levels increased in activated microglia without an increase in protein levels. In our study, we also did not find changes in GDNF release following the LPS activation of microglia, and after the treatment with the antidepressants (10 μM) we found there were no increases in GDNF protein levels. It is possible that GDNF mRNA levels are increased in microglia following antidepressant treatments, but these levels were not measured. The antidepressant treatments did not alter GDNF release from microglia; therefore we did not expect this trophic factor to be a main factor in mediating neuronal viability in co-culture.

There is a paucity of studies measuring the release of IL-4 from activated microglia. The majority of studies on IL-4 focus on its anti-inflammatory properties in attenuating the phenotype of activated microglia. For instance, Kitamura et al. (2000) showed that the pre-treatment of LPS-activated microglia with IL-4 significantly reduces the mRNA expression levels of various inflammatory factors including cyclooxygenase 2 (COX-2), iNOS, and TNF-α. In our study, we measured the release of IL-4 from LPS-activated microglia and following antidepressant treatments. We found that LPS or the antidepressants did not alter the release of IL-4 in microglia. Soliman et al. (2012) also

measured the release of IL-4 from LPS-activated microglia and they reported increases in IL-4 mRNA following activation, but no increases in protein levels. Similar to our GDNF data, it is possible that IL-4 mRNA levels are increased in microglia following LPS or antidepressant treatments, but these levels were not measured. The antidepressant treatments did not alter IL-4 release from microglia; therefore, we did not expect this trophic factor to be a main factor in mediating neuronal viability in co-culture.

There are studies that measure the *in vivo* expression levels of BDNF following treatments with an antidepressant (Lee et al. 2011; Arakawa et al. 2012). For instance, Lee et al. (2000) found that escitalopram (the therapeutically active enantiomer of citalopram) treatment increased the levels of BDNF protein levels *in vivo* following ischemic insult; however, this study did not investigate the source of BDNF increase. To my knowledge no study has measured the release of BDNF from microglia following antidepressant treatments.

We found that LPS increases the release of BDNF from microglia, a result that is supported by other reports in the literature (Miwa et al. 1997; Gomes et al. 2013). Following antidepressant treatments, we found that imipramine, clomipramine, and fluoxetine increased the release of BDNF from non-activated microglia, whereas the addition of LPS with these antidepressants had no additional effect. Treatment with phenelzine or citalopram did not affect the release of BDNF from microglia and did not significantly alter the release of BDNF from LPS-activated cultures. These results do not correlate with the co-culture data. In co-culture, activated microglia that are pre-treated with fluoxetine or citalopram attenuate the losses in viability of OGD-injured cortical

neurons, but citalopram did not alter BDNF release from activated microglia. In addition, impramine and clomipramine both increased BDNF release from microglia, but these antidepressants did not attenuate the reduction in neuronal viability. It can be argued that BDNF has a duel role in neuroprotection depending on the receptor the factor binds to. BDNF binds to at least two types of receptors on the surface of cells, namely the tyrosine kinase B receptor (Trk B) and the low affinity nerve growth factor or p75 receptor (Patapoutian and Reichardt 2001). Through the Trk B receptor, BDNF helps support the survival of existing neurons and supports the growth and differentiation of developing neurons and synapses (Huang and Reichardt 2001). Through the p75 receptor, it has been shown the BDNF signals neurons to die via apoptosis (Kenchappa et al. 2010; Cortes et al. 2012). It is possible that the OGD-injured neurons increase the expression of the p75 receptor (Tonchev 2011) and the increase in BDNF release from LPS-activated microglia causes the additional losses in neuronal viability in co-culture, but evidence from our data disagree with this suggestion. First, imipramine, clomipramine, and fluoxetine are all antidepressants that increased BDNF release from non-activated microglia in our study, but did not cause a loss in neuronal viability. Second, citalopram did not significantly alter the expression of BDNF from LPS-activated microglia, but attenuated the loss in neuronal viability in co-culture. Lastly, neutralization of BDNF released from LPS-activated microglia with an anti-BDNF scavenging antibody had no effect on the viability of OGD-injured neurons compared to those co-cultures containing LPS-treated microglia with no BDNF scavenger. If BDNF were an important factor in decreasing neuronal viability, we would expect attenuation in the neuronal viability loss following the neutralization of BDNF from LPS-activated microglia. To rule out trophic

actions of microglial-derived BDNF on neuronal viability, BDNF was neutralized in the co-cultures containing fluoxetine and citalopram-treated microglia. If BDNF were important in preventing the loss in neuronal viability, neutralizing BDNF should abolish the neuroprotective effects of fluoxetine- and citalopram-treated microglia. We found that in the conditions of fluoxetine- or citalopram-treated microglia in co-cultures with OGD-injured neurons, neutralizing the BDNF released from these microglia had no effect on the viability these neurons. With these results, we did not expect BDNF to be a main factor released from microglia in mediating neuronal viability in co-culture.

6.4.2. Amino acid release from microglia

The next step was to measure the release of amino acids from microglia following LPS activation and antidepressant treatments. Previous reports have shown that glutamate and D-serine are released from activated microglia (Patrizio and Levi 1994; Wu et al. 2004; McMullan et al. 2012), but no study has examined the release of these amino acids following antidepressant treatment. We found that LPS increased glutamate and D-serine release from microglia, but fluoxetine or citalopram treatment attenuated this release. Treatment with the other antidepressants (imipramine, clomipramine, phenelzine, or TCP) did not attenuate the release of glutamate or D-serine from LPS-activated microglia. The release of seven other amino acids (L-serine, glycine, glutamine, aspartate, taurine, alanine, and GABA) from microglia was also measured following LPS and/or antidepressant treatments and we found no significant differences in the release of these amino acids across all conditions. These results let us to hypothesize that glutamate

and/or D-serine release from LPS-activated microglia are main factors that mediate the viability of OGD-injured neurons.

We used various antagonists or inhibitors to neutralize the effects of many factors including AG (iNOS inhibitor), anti-TNF-α antibody (TNF-α scavenger), L-701 (NMDA glycine site inhibitor), and MK-801 (NMDA receptor inhibitor). We performed control experiments to verify the inhibitory or scavenger functioning of each antagonist before using these compounds in co-culture. Inhibition in the production of NO from LPS-activated microglia with AG did not attenuate the loss in viability of injured cortical neurons in co-culture, but scavenging of TNF-α did attenuate this loss. A similar result was found in a previous study, as Kaushal and Schlichter (2008) found that the inhibition of iNOS in microglia with the inhibitor S-methylisothiourea did not attenuate cortical neuronal death following OGD injury in co-culture. When interpreting these results, we must be cautious. NO is a highly unstable molecule with a half-life of a few seconds (Hou et al. 1999), and NO has effects on targets in close proximity of its release. In our study, NO levels are measured indirectly by quantifying the levels of nitrites, the by-product of NO instability (Green et al. 1982) and do not measure NO levels directly. Furthermore, co-cultures contain fixed microglia and cortical neurons that cannot alter their distances with respect to one another and it is speculative whether the release of NO from microglia can affect the neurons before its breakdown. It is questionable if the iNOS inhibitor used in our study was even necessary since the NO produced may not reach the neurons; however, there is an additional manner in which NO may induce neuronal death. NO can react with the free radical superoxide to form peroxynitrate.

Peroxynitrate is a highly reactive species which can compromise the survival of a cell by reacting with various components including lipids, amino acid residues, and DNA bases, leading to cell toxicity (O'Donnell et al. 1999). Peroxynitrate affects protein structure and function and has the potential to cause changes in the catalytic activity of enzymes and impair cell signal transduction (Pacher et al. 2007). It was imperative to use the iNOS inhibitor to rule out the production of NO-derived peroxynitrate in co-culture.

A contradicting study reported that LPS administration increased the amount of glutamate uptake by microglia and increased the expression of GLT-1, a glutamate transporter (Persson et al. 2005). Our study demonstrated that microglial glutamate release, rather than uptake, was increased in the presence of LPS. One possible explanation is that Persson et al. (2005) used a type of medium that contained little glycine and practically no glutamine. Microglial glutamate release *in vitro* has been shown to be dependent on glutamine and glycine in the medium (Patrizio and Levi 1994). Glutamate can be formed from glutamine or from glycine by the enzymes glutaminase and glycine transaminase (Grant et al. 2006), respectively, and low levels of glutamine or glycine in medium may result in a decrease in cellular levels of glutamate and may be the cause a compensatory increase of GLT-1 expression on microglia. Furthermore, it is more likely that *in vitro* conditions containing glutamine and glycine in the medium better reflect *in vivo* conditions.

When NMDA receptors on the injured cortical neurons were inhibited with MK-801 or L-701 in the co-cultures, we found attenuation in the loss of neuronal viability when

cultured with LPS-activated microglia, similar to the results following fluoxetine and citalopram treatment of microglia. MK-801 showed greater attenuation in this viability loss compared to L-701, but both were significantly greater compared to cultures consisting of LPS-treated microglia with no inhibitors. These results demonstrate that the NMDA receptors on the cortical neurons are highly involved in mediating neuronal viability in the co-culture. Though these studies show the NMDA receptor is involved in mediating this neuronal viability, we cannot be certain that the glutamate and D-serine that stimulate these receptors come entirely from the LPS-activated microglia. It is possible that glutamate and/or D-serine release from microglia stimulates NMDA receptors on the neurons, causing these neurons to release more glutamate, and in turn causing the further reduced viability. Another possible scenario is that glutamate release from LPS-activated microglia may be induced by TNF-α. Takeuchi et al. (2006) have shown that TNF-α released from activated microglia can induce the release of glutamate from these same cells in an autocrine manner. They showed that this glutamate is released from gap junction-associated hemichannels on the surface of microglia rather than through glutamate transporters, and this TNF-α induced glutamate release is toxic to cortical neurons. Our findings do not agree entirely with these previous results. We did show significant attenuation in neuronal viability loss following scavenging of TNF-α released from LPS-activated microglia in co-cultures, but this was not to the extent seen with the NMDA receptor antagonist (MK-801 and L-701) groups. This suggests that glutamate release is not fully dependent on the autocrine effects of TNF-α. In addition, this result may suggest that glutamate release from microglia in co-culture can occur through both glutamate transporters and hemichannels, where hemichannel release is

mediated by TNF-α signaling. This may explain the partial attenuation observed after TNF-α scavenging, as glutamate can be released through transporters. One experiment that can be performed to test this idea is to measure the amount of glutamate in the medium of activated microglia following treatments with specific inhibitors for the glutamate transporters or hemichannels. This experiment should be carried out with caution, since it is possible that inhibiting one release mechanism may induce compensatory release from microglia using the alternate mechanism.

Recently, Malkesman et al. (2012) have reported that acute D-serine treatment produces antidepressant-like effects in rodents based on various behavioral tests. Our data oppose this finding as we show that D-serine released from microglia is a main factor in reducing the viability of injured neurons and we also show that citalopram and fluoxetine both decrease this D-serine release. Several questions surface from the study by Malkesman et al. (2012). Dysfunctional glutamatergic signaling has been linked to clinical depression and to rodents displaying depressive-like behaviors such as anhedonia and social defeat (Steiner et al. 2011; McCarthy et al. 2012). Malkesman et al. (2012) show that the acute treatment with D-serine may improve depressive symptoms in rodents for the short term (24 hrs), but did not investigate the behavioral outcomes over an extended period of time (over several weeks). It is possible that the depressive-like behavior in these rodents resurface if measured over several weeks and that they require chronic D-serine treatment. This chronic treatment with D-serine may lead to excitotoxic neuronal death mediated by the NMDA receptor; however, this idea is only speculative since it has not been investigated. A main difference between the studies conducted by Malkesman et al.

(2012) and our laboratory is that in their study, they induced depressive-like symptoms in rodents using stress paradigms including learned helplessness and the forced swim test, whereas we used an *in vitro* set-up investigating the effect of antidepressants on OGD-injured neurons. It is possible that D-serine has different effects on neurons with altered glutamate transmission compared to ischemic-injured neurons. Inducing stress in rodents can lead to altered glutamatergic neurotransmission (Marsden 2011; Sanacora et al. 2012) and in this model D-serine may be beneficial in restoring this activity, whereas in our model, cortical neurons were injured with OGD, and D-serine may become toxic to these neurons (Wu et al. 2007; Mustafa et al. 2010).

The antidepressant data in this study do not fully support the idea that TNF-α is a main factor in reducing neuronal viability in co-culture. Imipramine and TCP were both shown to attenuate TNF-α release from LPS-activated microglia, but when these treated microglia were placed in co-cultures, there was no significant attenuation in the microglial-induced neuronal viability loss. Together, these results suggest that NMDA receptors on the cortical neurons represent a principal factor that reduces the neuronal viability following co-cultures with LPS-activated microglia and that these receptors are activated by glutamate and/or D-serine released by microglia and possibly neurons. Another caution in this study is with the lack of astrocytes in these cultures since the presence of these cells would more closely mimic an *in vivo* environment. Astrocytes are important in controlling the levels of extracellular glutamate by taking it up through transporters (Deng et al. 2012; Verbich et al. 2012). A possibility is that astrocytes may take up the glutamate released from LPS-activated microglia and thereby prevent

neuronal toxicity in co-culture; however, there is evidence that LPS activates astrocytes through the TLR4 receptor (Gorina et al. 2011) and this activation can lead to the release of glutamate from these cells (Brown and Bal-Price 2003). Thus, activated astrocytes may lead to further neuronal toxicity in addition to the effects of microglia.

6.5. What is the mechanism behind the attenuation in the release of these main players from activated microglia following antidepressant treatment?

Our next step was to investigate the possible mechanism behind fluoxetine's and citalopram's effects on inhibition of glutamate and D-serine release from LPS-activated microglia. Our results showed an interesting trend in that fluoxetine-treated microglial cultures contained significantly lower protein levels and reduced MTT cell viability compared to non-treated cultures 24 hrs post-treatment. These effects were not seen with cultures treated with any other antidepressant or LPS in this study. We hypothesized that fluoxetine causes microglial apoptosis and this mechanism may be one possibility of how this drug prevents these cells from releasing glutamate or D-serine following LPS-activation.

6.5.1. Antidepressant effects on microglial apoptosis

We performed a variety of experiments to demonstrate that fluoxetine induced microglial apoptosis. First, the expression of cleaved-caspase 3 (apoptotic marker) was measured with Western blotting following various antidepressant treatments. Caspase 3 is cleaved by other proteases such as caspases 8, 9, or 10, and this cleaved active enzyme goes on to

execute apoptosis. We found that fluoxetine significantly increased the expression of cleaved-caspase 3. We also tested the effects of imipramine (TCA) and phenelzine (MAOI) and found no effects of these antidepressants on the expression levels of cleaved-caspase 3 or caspase 3 in microglia. Second, using fluorescent labeling, microglial cultures treated with antidepressants were co-labeled with Iba1 (a microglial marker) and cleaved-caspase 3 (an apoptotic marker) to visualize apoptotic microglia. Fluoxetine-treated microglia expressed a greater number of co-labeled cells, whereas imipramine and phenelzine showed no effect compared to non-treated cultures. Finally, microglia cultures underwent live-dead fluorescent staining testing for membrane integrity following antidepressant treatment. The live stain is permeable and penetrates all cells, whereas the dead stain is impermeable and only penetrates those cells with compromised cellular membranes. Fluoxetine-treated microglial cultures expressed a greater number of co-stained cells, whereas imipramine and phenelzine showed no effect compared to non-treated cultures. In summary, we found that fluoxetine induced the apoptotic death of microglia, and this is a possible mechanism by which this drug attenuates the release of glutamate from these cells; however, in addition to inducing apoptosis, it may be possible that fluoxetine can block glutamate transporters or hemichannels to prevent glutamate release (Takeuchi et al. 2006) and microglial apoptosis can be a separate effect of this antidepressant. Therefore, we cannot conclude the exact mechanism behind the attenuation of glutamate release from microglia following treatment with fluoxetine. We obtained some unexpected results from experiments measuring the effects of citalopram on microglial apoptosis. We have demonstrated that both fluoxetine and citalopram attenuate the release of glutamate and

D-serine, and that fluoxetine induces microglial apoptosis; therefore, we hypothesized that citalopram would also induce microglial apoptosis. Citalopram-treated microglial cultures did not show any changes in the expression levels of cleaved-caspase 3 or caspase 3 with Western blotting or immuno-fluorescence and had no effect on live-dead staining compared to non-treated controls. These unexpected results led to questions that still require investigation. There must be an alternative explanation other than inducing apoptosis as to how citalopram attenuates the release of glutamate and D-serine from cultured microglia including the blocking of glutamate transporters or hemichannels (Takeuchi et al. 2006); however, these mechanisms require further investigation.

6.5.2. Mechanisms of D-serine release

In astrocytic cell cultures, D-serine has been shown to be released from vesicles through exocytosis following the stimulation of the 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid (AMPA) or metabotropic glutamate receptors and this exocytosis is dependent on calcium influx and on the SNARE complex involved in transmitter release (Mothet et al. 2005). In addition, Rosenberg et al. (2010) found that non-vesicular D-serine can be released from astrocytes through volume-regulated anion channels. Following AMPA receptor stimulation, astrocytes swell and enlarge, causing these channels to open, allowing D-serine release (Haas and Erdo 1991). These mechanisms of D-serine release have not been studied in microglia, and future experiments can focus on these mechanisms of release since these cells can also release vesicles mediated by the SNARE complex (Turola et al. 2012). For instance, we can inhibit the SNARE complex, disturb the proton gradient across vesicular membranes with bafilomycin or

concanamycin, or treat microglial cultures with calcium chelators such as BAPTA or EGTA and then measure D-serine release following LPS activation using HPLC. Decreases in D-serine levels following these manipulations would indicate that D-serine is released via exocytosis and is mediated through the SNARE complex and/or calcium influx. If we find that D-serine is released in this fashion, it is possible that fluoxetine or citalopram may inhibit this release either by interfering with the SNARE complex or with calcium influx. If the release of D-serine is not decreased from LPS-activated microglia following inhibition of SNARE and calcium chelation, we can then block the volume-regulated anion channels on LPS-activated microglia with various inhibitors including flufenamic acid (FFA), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), or 4-[(butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-iden-5-yl)oxy]butanoic acid (DCPIB) (Rosenberg et al. 2010). If D-serine release is attenuated with these inhibitors it is possible that fluoxetine or citalopram may inhibit this release by interfering with the volume-regulated anion channels on microglia.

6.5.3. Norfluoxetine and fluoxetine effects on microglial apoptosis

After oral administration in a human, fluoxetine is mainly excreted in urine, with less than 10% excreted unchanged or as fluoxetine *N*-glucuronide (Benfield et al. 1986). The remaining fluoxetine is metabolized into norfluoxetine and other non-active metabolites primarily by the enzymes CYP2D6 and CYP2C9 in the liver (Whirl-Carrillo et al. 2012). Since the majority of fluoxetine is metabolized into norfluoxetine in the human, we investigated if norfluoxetine also induced apoptosis of microglia in culture. We found that norfluoxetine increased the expression of the apoptotic marker cleaved-caspase 3

while decreasing caspase 3, increased the number of microglia co-labeled with cleaved-caspase 3 and Iba1 as measured using immunofluorescence, and increased the number of cells co-labeled with both the live and dead stains. In addition, norfluoxetine decreased the levels of protein within microglial cell lysates and reduced MTT cell viability. These results raised an important issue in that microglia may be converting fluoxetine into norfluoxetine, and that norfluoxetine may be the drug that induces apoptosis. To test this, we treated microglia with fluoxetine and measured the amounts of norfluoxetine and fluoxetine in the cell lysates using GC-MS. We measured insignificant amounts of norfluoxetine in these lysates, demonstrating that the cultured microglia did not convert fluoxetine into norfluoxetine. We conclude that both fluoxetine and norfluoxetine can induce the apoptotic cell death of microglia but that norfluoxetine is not formed by microglia *in vitro*.

Fluoxetine and norfluoxetine may induce apoptosis by one of two primary pathways, the extrinsic or intrinsic pathway (Slee et al. 2001). To investigate if these antidepressants initiated the extrinsic pathway, we measured the expression levels of cleaved-caspase 8 and caspase 8 in microglial cell lysates. Caspase 8 associates with death receptors that are members of the TNF receptor gene superfamily and can translocate within the cell after cleavage to activate other caspases (Kischkel et al. 1995; Locksley et al. 2001). We found that fluoxetine- or norfluoxetine-treated cells expressed no cleaved-caspase 8, similar to non-treated controls; however, this may be due to a defective antibody used for these studies. When we treated microglia with 20 ng/ml of TNF-α, a positive control of extrinsic apoptosis, there was no expression of cleaved-caspase 8. These results may

demonstrate that the antibody for cleaved-caspase 8 was defective. We measured the expression levels of caspase 8 and found that fluoxetine, staurosporine, and TNF-α-treated microglia expressed lower levels of caspase 8 compared to non-treated controls. Norfluoxetine did not decrease caspase 8 levels but decreased caspase 3 levels while increasing cleaved-caspase 3 expression. It is likely that fluoxetine induces extrinsic apoptosis based on the decrease in caspase 8 levels; however, we cannot conclude this without a functional cleaved-caspase 8 antibody. Norfluoxetine may induce intrinsic apoptosis since the drug shows effects on caspase 3 levels and no effects on caspase 8 levels. One strange result in this experiment is that TNF-α treatment (positive control of extrinsic apoptosis) did not show increases in cleaved-caspase 3 expression, but does decrease caspase 3 levels in microglia. One possible explanation for this is that 20 ng/ml of TNF-α may be a strong inducer of apoptosis, and that the cleaved-caspase 3 associates with other downstream caspases, rapidly leaving behind little free floating cleaved-caspase 3 to be detected. One way to determine if this is the case is to vary the time of TNF-α treatment of microglia from 24 hrs to a lower time point and then measure the expression of cleaved-caspase 3. At a lower time point, cleaved-caspase 3 may be available to measure following TNF-α treatment; however, staurosporine is another strong inducer of apoptosis, and microglia treatment with this compound shows an increase in cleaved-caspase 3 expression following 24 hrs treatment. Another explanation could be that the concentration of TNF-α is not sufficient to induce apoptosis, but there is evidence against this point. The concentration of TNF-α used in this study (20 ng/ml) decreased caspase 8 and caspase 3 levels, but if this concentration was not sufficient, we likely would not see these changes.

There is a experimental design that would strengthen the data in this study. We can inhibit the death receptors on microglia with compounds such as Fas apoptosis inhibitory molecule (FAIM) or a TNF receptor antagonist (Sole et al. 2004; Gururaja et al. 2007) and determine if fluoxetine and norfluoxetine still induce apoptotic death. Based on the results in caspase 8 expression, we would hypothesize that the antagonism of the death receptors on microglia would inhibit apoptosis induced by fluoxetine but not norfluoxetine.

6.5.4. Beneficial roles of SSRI antidepressants following stroke

We have shown that glutamate and D-serine release from LPS-activated microglia are main factors that reduce the viability of OGD-injured cortical neurons. Fluoxetine is an antidepressant that decreases the release of glutamate and D-serine from LPS-activated microglia and attenuates the loss in neuronal viability. We have shown that fluoxetine induces apoptosis in microglia and this can be the mechanism behind the attenuation in glutamate and D-serine release. In an ischemic region of the brain, microglia are in a highly reactive and proliferative state as they release a variety of inflammatory and toxic factors, and anti-inflammatory drugs such as minocycline and fluoxetine have been shown to protect against these insults (Stoll et al. 1998; Schroeter et al. 1999; Yrjanheikki et al. 1999; Lim et al. 2009). The frequency of depression following a stroke is highest after one month and the risk remains elevated for several years, and the treatment with SSRI antidepressants has been shown to reduce the incidence of depression (Gothe et al. 2012; Loubinoux et al. 2012). For instance, the SSRI fluvoxamine has been shown to

alleviate the diminished mood and sleep disturbances in patients with post-stroke depression (Sunami et al. 2012). In addition, chronic treatment with the SSRI citalopram has been shown to delay the degeneration of dopaminergic neurons in the midbrain, prevent striatal atrophy, and reduce the behavioral symptoms in rodents following MCAo (Kronenberg et al. 2012). An idea that stems from our study is that the SSRIs fluoxetine and citalopram attenuate the reactive and proliferative state of microglia by reducing their release of inflammatory and toxic factors, most importantly glutamate and D-serine, and ultimately reducing neuronal toxicity after an ischemic insult. In addition, the SSRI fluoxetine may induce microglial apoptosis to help regulate the numbers of reactive and proliferatve microglia in a region of ischemic insult. The effects of these SSRIs may be beneficial following stroke, but there is evidence supporting a detrimental role of these SSRIs in the absence of an ischemic insult.

6.5.5. Detrimental role of SSRI prior to a stroke

In a 9-year follow up study, Li et al. (2012) found that patients diagnosed with depression and prescribed SSRI antidepressants had higher rates of stroke in later life and this finding has been supported by a number of studies (Bushnell 2011; Andrews et al. 2012; Castro et al. 2012; Simmons et al. 2012). Smoller (2011) reported that depressed postmenopausal women had a 45% increased risk in stroke if prescribed SSRI antidepressants and the risk of fatal stroke was doubled. In support of this, Ried et al. (2011) reported that depressed patients prescribed SSRI antidepressants were three times more likely to die following a stroke. The data in our studies may help explain these findings. In the absence of microglia, tissue damage has been shown to be exacerbated

following a stroke, supporting a beneficial role of microglia (Lalancette-Hebert et al. 2007). We found that fluoxetine induces microglial apoptosis *in vitro* and if fluoxetine had this same effect clinically by causing cell death of microglia in patients, then we would expect less microglial availability in times of insult and stress. This reduced availability in microglia may cause the increased risk of fatal stroke in these depressed patients. We can investigate this idea using an *in vivo* setup with rodents as the subjects. We can chronically treat a group of rodents with various SSRIs, over a period of several weeks and have a separate control group that has been treated with saline (no drug). We can then induce an ischemic stroke in these rodents using MCAo. We would then test for various locomotor and behavioral deficits in these rodents after a recovery period and compare the deficits between the two groups. We can also remove the brains of these rodents and label for microglia and measure infarct volumes. We would hypothesize that rodents given SSRIs, specifically fluoxetine, would show greater locomotor and behavioral deficits, reduced numbers of microglia, and enlarged infarct volumes compared to the saline controls.

In our study, we found that citalopram did not induce microglial apoptosis, but others have found that citalopram does increase the susceptibility of fatal stroke in depressed patients (Bushnell 2011; Mikami et al. 2011). One limitation in our study was that we only investigated the effects of citalopram treatment on microglial apoptosis for 24 hr. It is possible that longer or chronic treatments of citalopram may eventually induce microglial apoptosis. Another limitation in our study is that we did not investigate the effects of the metabolites of citalopram on the induction of microglial apoptosis.

Citalopram is metabolized into desmethylcitalopram by the enzymes CYP2C19 and CYP3A4 and desmethylcitalopram is further metabolized into didesmethylcitalopram by CYP2D6 in the liver (Sangkuhl et al. 2011). These metabolites are significantly less potent reuptake inhibitors of 5-HT, but we cannot rule out the possibility that these metabolites induce microglial apoptosis. Although these metabolites cross the BBB poorly compared to their parent citalopram (Sangkuhl et al. 2011), studies investigating the effects of these metabolites on microglial apoptosis should be performed.

6.6. Concluding statement

In our studies, we demonstrated a number of things. First, we demonstrated that a variety of antidepressants across three separate classes attenuate the release of NO, TNF-α, and IL-1β from activated microglia. Second, we demonstrated that the SSRI antidepressants fluoxetine and citalopram were the only antidepressants of the ones tested to reduce the loss in the viability of ischemic-injured cortical neurons through their anti-inflammatory effects on microglia. Previous studies have also reported this finding with citalopram, but we are the first to report this with cortical neurons. Third, we demonstrated that glutamate and D-serine released from microglia are important players in reducing this neuronal viability and both fluoxetine and citalopram attenuate the release of these amino acids from LPS-activated microglia. Finally, we demonstrated that fluoxetine induces microglial apoptosis and this may be a mechanism behind the attenuated release of glutamate and D-serine from LPS-activated microglia. Although we have reported these results, there are limitations to our studies that need to be considered. For instance, a major limitation is that we did not perform *in vivo* experimentation to corroborate our *in*

vitro studies. We have given an example of a feasible *in vivo* study that investigates if prior treatment with antidepressants influences recovery following an ischemic stroke in rodents by inducing microglial apoptosis. The *in vivo* studies would strengthen the data obtained from the *in vitro* studies in this thesis. Despite these limitations, it is important to determine the beneficial or detrimental outcomes following SSRI treatments. Other than fluoxetine, norfluoxetine, and citalopram, many other SSRIs or SNRIs including sertraline, paroxetine, and venlafaxine are prescribed to depressed patients. These antidepressants should be tested to determine their effects on microglial apoptosis and future susceptibility to stroke. In conclusion, the treatment of post-stroke depression with SSRIs may be beneficial through the attenuation of an exaggerated microglial response; however, treatment of depression with SSRIs in the absence of an insult may be detrimental over time by causing microglial apoptosis and leaving a reduced number of microglia in times of an insult such as stroke. These hypotheses need to be investigated further.

6.7. References

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